University of Alberta

Somatostatin Gene Expression in the Brain of Goldfish.

by

Carla Jane Otto

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Masters of Science

in

Physiology and Cell Biology

Department of Biological Sciences

Edmonton, Alberta

Fall 1998

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-34399-5

Canada

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Somatostatin Gene Expression in the Brain of Goldfish** submitted by **Carla Jane Otto** in partial fulfillment of the requirements for the degree of **Master of Science** in **Physiology and Cell Biology**.

(Dr. R.E. Peter, Supervisor)

(Dr. J.P. Chang, Committee Member)

(Dr. S. Harvey, Committee Member)

(Dr. C. Strobeck, Chairperson)

Sept 11, 1998

Abstract

A novel somatostatin (SS) cDNA, PSS-I^2, which encodes for the mature peptide [Pro^2]SS-14, was sequenced from the brain of goldfish. The three known somatostatin genes, PSS-I^1, PSS-I^2 and PSS-II, were expressed in unique regions of the brain and body, and are believed to originate from unique ancestral genes. [Pro^2]SS-14 inhibited both basal and stimulated growth hormone (GH) secretion from perfused pituitary fragments. This effect is similar to that of SS-14, the mature peptide produced from PSS-I^1. PSS-I^2 and PSS-II mRNA expression in the forebrain varied seasonally, inversely to serum GH levels previously measured. A small elevation of PSS-I^1 expression occurred only in females at sexual maturity, when GH secretion is high, suggesting a limited role of SS-14 in seasonal GH control. Estradiol and testosterone implantation in female fish increased the expression of all three PSS genes, while dopamine differentially regulates PSS gene expression through D_1 and D_2 receptors with seasonal differences.

Acknowledgement

I would like to thank my supervisor Dr. Richard E. Peter for sparking my interest in fish endocrinology four years ago and supporting me, both intellectually and financially, through this Master's degree in his lab. I would also like to thank Carol Nahorniak for her comments on techniques and life, running at lunch, and cleaning up my messes. Thanks also to my committee members John Chang, and Steve Harvey for their comments on my research. I would also like to thank my family for their support and enthusiasm though all my schooling; Eugene Chomey helping me learn molecular biology and for reminding me to enjoy graduate life; Jim Johnson for coming down to the fourth floor to ask me question about what I was doing; Bruce Wakeford for keeping my goldfish alive and swimming; and my lab mates Wei Lin, Helen Volkoff and Pierre Peyon for their comments on my project.

I was supported by Dr. RE Peter's Natural Sciences and Engineering Research Council of Canada (NSERC) grant, and the Department of Biological Sciences as a Laboratory instructor. Funding for travel to the Canadian Society of Zoologists conference was provided by the Faculty of Graduate Studies and Research and the Department of Biological Sciences.

Table of Contents

Chapter 1 Introduction 1

Somatostatin in Mammals

Peripheral Somatostatin 1

Peptide Processing 4

Somatostatin Metabolism 5

Somatostatin Receptors 5

Somatostatin Distribution in the CNS 6

Somatostatin Gene 8

Somatostatin Gene Expression 8

Growth Hormone Feedback Axis 9

Daily cycle of growth hormone 9
Short-loop feedback 10
Long-loop feedback 11
GHRH and somatostatin hypothalamic interactions 12

Factors Affecting Brain Somatostatin Expression and Secretion 13

SS and Sex Steroids 13
Dopamine 15

Somatostatin in Teleosts

Forms of Somatostatin Found in Lower Vertebrates 16

Distribution of Somatostatin Immunoreactivity in the Brain 17

Somatostatin and Growth Hormone in Teleosts 18

Sex Steroid Interactions in Teleosts 19

Intracellular mechanisms for Somatostatin inhibition of GH 21

Objectives of Study 22

CHAPTER 2 Material and Methods 25

Molecular Cloning of cDNA Encoding PSS-I2

Screening of cDNA Library 25

Subcloning and sequencing 26

Isolation and Sequencing of Partial Gene for PSS-II 26

Differential Expression of the Three Somatostatin Genes

Preparation of Total RNA 27

Preparation of messenger RNA from Pituitary Cells 28

Probe Synthesis for Southern and Northern Analysis 29

RT-PCR Assay and Southern Blot Analysis 30

Northern Blot Analysis 30

Functional Analysis of [Pro2]SS-14 by *in vitro* Perifusion of Pituitary Fragments.

Perifusion of Pituitary Fragments 31

Data Analysis 32

Regulation of Somatostatin Gene Expression

Reagents and Test Substances 33

Treatments
Injections 34
Steroid Implantation 34

Seasonal Sampling 34

Quantitative Analysis of mRNA Levels 35

Figures 36

CHAPTER 3 Results 38

Complementary DNA Encoding PSS-I2

Goldfish Preprosomatostatin-I2 38

Phylogenetic Analysis of the Known PSS Coding Sequences 39

Partial Sequence for PSS-II Gene 39

Differential Expression of PSS Genes in Different Tissues and Brain Regions

Body 40

Pituitary 40

Brain 40

Functional Characteristics of [Pro^2]SS-14

Inhibition of pituitary GH secretion 41

Effect on sGnRH-stimulated GH release 41

Effect on D1 Agonist-Stimulated GH Release 42

Regulation of Somatostatin Gene Expression

Seasonal Alteration in Somatostatin Gene Expression

PSS-I^1 43

PSS-I^2 43

PSS-II 44

Regulation of Somatostatin Gene Expression by GnRH 44

Dopaminergic Regulation of Somatostatin Gene Expression

Effect of catecholamines on SS gene expression 44

Effect of Dopamine Agonists and Antagonists on PSS mRNA Expression in the Forebrain

1. Apomorphine 45
2. D_1 receptors 45
3. D_2 receptors 46

Regulation of Somatostatin Gene Expression by Gonadal Steroids 47

Figures 48

Chapter 4 Discussion

Phylogenetic Origins of Goldfish Somatostatin Genes 72

Distribution 74

Biological Activity 77

Seasonal Variation 79

Sex Steroids 81

sGnRH Effects on PSS mRNA expression in the Forebrain of Goldfish 84

Dopaminergic Effects on PSS mRNA Expression in the Forebrain of Goldfish 84

General Conclusions 88

Literature Cited 91

Appendix 1 110

Appendix 2 112

List of Tables

Table 2-1

Primers used for sequencing the partial gene sequence encoding preprosomatostatin-II from the liver of goldfish. 27

Table 2-2

Primers used in PCR for synthesis of the three PSS probes. 29

Table 3-1

Gonadal Somatic Index (GSI) of goldfish collected in April, July, and December. 44

List of Figures

Fig. 1-1	Processing of the Rat SS gene from gene to mature peptide products.	24
Fig. 2-1	A schematic diagram of the goldfish brain divided into 6 distinct regions.	36
Fig. 2-2	A schematic diagram of the goldfish brain defining the forebrain region.	37
Fig. 3-1	cDNA sequence for PSS-I^2.	48
Fig. 3-2	A phylogenetic tree describing the relationships between coding regions of all known SRIF mRNA regions.	49
Fig. 3-3	PCR products of genomic DNA amplified with specific primers for PSS-I^1, PSSI^2 and PSS-II, separated on a 1% agoarose gel.	50
Fig. 3-4	The partial goldfish genomic sequence encoding PSS-II.	51
Fig. 3-5	RT-PCR and Southern analysis of 6 regions of the body with probes and primers specific for PSS-I^1, PSS-I^2 and PSS-II.	52
Fig. 3-6	Northern analysis of five region of the brain and the pituitary.	53
Fig. 3-7	RT-PCR products amplified from pituitary cell mRNA with specific primers for PSS-I^1, PSS-I^2 and PSS-II.	54
Fig. 3-8	Southern analysis of five regions in the brain	55
Fig. 3-9	The effects of SS-14 and [Pro^2]SS-14 on pituitary GH secretion.	56
Fig. 3-10	Effects of [Pro^2]SS-14 on sGnRH-stimulated GH release from perifused pituitary fragments of goldfish.	57
Fig. 3-11	Effects of [Pro^2]SS-14 on dopamine-stimulated GH release form perifused pituitary fragments of goldfish.	58
Fig. 3-12	Seasonal (gonadal state) effects on the expression of PSS mRNA in the forebrain of goldfish.	59
Fig. 3-13	Effects of sGnRH by i.p. injection on PSS mRNA expression in the forebrain of sexually regressed male and female goldfish.	60
Fig. 3-14	Effect of α-MPT i.p. injection, on PSS mRNA expression in the forebrain of sexually regressed male and female goldfish.	61
Fig. 3-15	Effect of Dopamine agonist apomorphine on PSS mRNA expression in the forebrain of sexually regressed male and female goldfish.	62

Fig. 3-16 Effects of SKF 38393, a D1 agonist, on PSS mRNA expression in the forebrain of sexually regressed male and female goldfish. 63

Fig. 3-17 Effects of SKF 38393, a D1 agonist, on PSS mRNA expression in the forebrain of sexually recrudescent male and female goldfish. 64

Fig. 3-18 Effects of Dopamine receptor agonists and antagonists on PSS mRNA expression in the forebrain of sexually recrudescent male and female goldfish. 65

Fig. 3-19 Effects of LY 171 555, a D2 agonist on PSS mRNA expression in the forebrain of sexually recrudescent male and female goldfish 66

Fig. 3-20 Effects of LY 171 555, a D2 agonist, on PSS mRNA expression in the forebrain of sexually mature female and male goldfish. 67

Fig. 3-21 Effects of LY 171 555, a D2 agonist, on PSS mRNA expression in the forebrain of sexually recrudexcent female goldfish. 68

Fig. 3-22 Effects of LY 171 555, a D2 agonist, on PSS mRNA expression in the forebrain of sexually recrudexcent male goldfish. 69

Fig. 3-23 Effects of Estradiol and Testosterone on PSS mRNA expression in the telencephalic-preopitc area of sexually regressed female goldfish. 70

Fig. 3-24 Effects of Estradiol and Testosterone on PSS-I^{1} and PSS-II mRNA expression in the hypothalamus of sexually regressed female goldfish. 71

Fig. A1-1 PSS-I^{1} cDNA sequence. 110

Fig. A1-2 PSS-II cDNA sequence. 111

Fig. A2-1 Known Mature Peptide SS sequences from vertebrates. 112

List of Common Abbreviations

Abbreviation	Description
AA	amino acid
ACTH	adrenocorticotropic hormone
BBS	bombesin
Bp	base pairs
CAMP	cyclic AMP
CCK	cholysistokinin
CNS	central nervous system
cDNA	complimentary DNA
DA	dopamine
DNA	deoxyribonucleic acid
E2	estradiol
5-HT	serotonin
GH	growth hormone
GHRH	growth hormone releasing hormone
GLP	glucagon/glucagon like peptides
GnRH	gonadotropin-releasing hormone
GTH-II	gonadotropin-II
IGF-I	insulin-like growth factor-I
ME	median eminence
MRNA	messenger RNA
MSH	melanotropin stimulating hormone
NE	norepinephrine
NPP	nucleus preopticus periventricularis
NAPv	nucleus anterioris periventricularis
NLT	nucleus lateral tuberis
NPY	neuropeptide Y
PACAP	pituitary adenylate cyclase activating polypeptide
PCR	polymerase chain reaction
PeV	periventricular nucleus
PPD	proximal parsdistalis
PSS	prosomatostatin
PVN	paraventricular nucleus
RNA	ribonucleic acid
RT-PCR	reverse transcription PCR
sGnRH	salmon gonadotropin-releasing hormone
SS	somatostatin
SSTR	somatostatin receptor
SON	supraoptic nucleus
T	testosterone
TRH	thyrotropin releasing hormone
TSH	thyrotropin stimulating hormone
i.p.	intraperitoneal
RIA	radioimmunoassay
VIP	vasoactive intestinal peptide

CHAPTER 1

Introduction

Somatostatin (SS) is a 14 amino acid peptide first isolated from ovine hypothalamus by Brazeau *et al.* (1973). At that time it was characterized as a hypothalamic polypeptide inhibiting secretion of immunoreactive pituitary somatotropin, also known as growth hormone (GH). Since then SS has been detected in the central nervous system, pancreas, stomach, small intestine, submandibular glands, kidneys, prostate, placenta and thyroid (Su *et al.* 1988). In rats, gut tissue accounts for 65% of total body SS, brain 25%, pancreas 5% and the remaining organs 5% (Patel *et al.* 1981).

Somatostatin in Mammals

Peripheral Somatostatin

Outside of the central nervous system (CNS), SS is important in a diverse array of organs. In the pancreas, SS decreases plasma levels of insulin and glucagon/glucagon-like peptides (GLP) as well as inhibiting their release from islet cells. Amylase secretion from acrinar cells of the pancreas is also inhibited (Brazeau *et al.* 1972, Epelbaum 1986, Gomez-Pan *et al.* 1975, Hellman and Lemmark 1969, Reichlin 1983). Furthermore, SS inhibits secretin, pepsin, vasoactive intestinal peptide (VIP), motilin and gastrin secretion in the gut *in vivo* (for review see Schally *et al.* 1978). SS also decreases smooth muscle contractility, and cell division (Reisine 1995). Important physiologic functions of SS in the kidney include mesangial cell contraction, glomerular prostaglandin synthesis, and phosphate, water and sodium excretion. Renal cells produce somatostatin and therefore kidney-derived somatostatin may regulate renal function in an autocrine/paracrine manner (Turman *et al.* 1997). SS in the placenta is involved in the regulation of placental blood flow critical

for fetal growth and development. It is also suggested that low maternal somatostatin levels are related to an efficient storage of nutrients in the fetoplacental unit, thereby leading to a high birth weight (Widstrom *et al.* 1989). Somatostatin receptors (SSTR), specifically SSTR4 subtype, have been identified in the human placenta (Caron *et al.* 1997). Immunoreactive SS in the prostate gland of human males is believed to play a strong role in pathological conditions, such as prostate cancer (Abrahamsson *et al.* 1986; Pekary *et al.* 1984). SSTR2 was found only in epithelial cells (EC) from a normal prostate. SSTR3 messenger RNA (mRNA) was undetectable in normal and malignant prostate EC, whereas SSTR4 and SSTR5 were present in both cell types (Sinisi *et al.* 1997). Receptors for SS are also present in the seminiferous tubules and Sertoli cells of the testis, suggesting an involvement in the male reproductive system (Zhu *et al.* 1998).

SS concentrations in the peripheral plasma are 50% lower than that found in the pituitary portal system (Plotsky 1991). The immunoreactive form of SS found in the peripheral plasma is also larger in molecular mass than SS-14 (Shoelson *et al.* 1986). The circulating form of SS may be an inactive form already degraded or the antibody may be able to adhere to other immunoglobulins circulating in the blood. Differences have also been detected between the levels of SS in systemic plasma and that in the pituitary portal circulation, as well as the two patterns of release (Plotsky 1991), which supports the suggestion that peripheral SS plays a minor role in the control of GH secretion. However, infusion of SS to plasma has repeatedly been shown to inhibit GH and GH levels rebound when SS infusion is suspended (Wass 1989). In the rainbow trout SS-14 has also been shown to inhibit GH secretion from pituitary cell cultures at concentrations lower than normal circulating SS-14 levels of regressed fish. The action of SS-14 in the plasma therefore remains unclear.

Somatostatin Peptide Processing

In mammals, SS is first synthesized as a 13 500 kD precursor molecule, preprosomatostatin (Goodman *et al.* 1983), with a 24 amino acid (AA) signal peptide at the N-terminal which is cleaved off in the lumen of the ER by a specific protease (Walter *et al.* 1984) leaving the prohormone, PSS. PSS is a 10 kD molecule of 92 AA which undergoes proteolitic cleavage at dibasic and monobasic sites. The prohormone is then processed enzymatically to form several mature products; SS-14, SS-28, SS-28 (1-12), ProSS(1-76), SS(1-63) and ProSS(1-10) otherwise known as antrin (Benoit *et al.* 1987; see Figure 1-1). Processing of the precursor in the human brain yields minimal quantities of SS-28(1-12) and high levels of ProSS(1-76), namely in cortical areas and in the bed nucleus of the stria terminalis. This is in contrast with findings in rat brain, where SS-28(1-12) is a predominant molecular form (for review see; Rabbani and Patel 1990). Antrin, which corresponds to ProSS(1-10), reaches its highest concentration in the antral portion of the stomach (117 ± 13 pmol/g wet weight), where it is found in secretory granules of delta cells (Benoit *et al.* 1990). Acid extracts of rat hepatic portal plasma contain a peptide similar or identical to antrin, indicating that the antral decapeptide circulates in blood (Ravazzola *et al.* 1989). Outside of the pancreas, all known SS-producing tissues are also rich in Pro-SS-(1-10) like-immunoreactivity (LI). Its concentration, however, is 1- to 4-fold lower than those of S-14 LI and S-28-(1-12) LI (Rabbani and Patel 1990). Biological activity is unknown for SS-28(1-12), ProSS(1-76), SS(1-63) and antrin.

Two PSS products have known SS biological activity, SS-14 and SS-28. They are processed from the C-terminal end of PSS. SS is recognized for its ring structure formed by a bond between Cys^3 and Cys^{14}. This ring is not necessary for biological activity, however, as demonstrated by functional linear analogs, although the specificity of action may be affected (Wass 1988). The AA sequence necessary for biological activity is Phe^7-Trp^8-Lys^9-Thr^{10}. SS-28 is an N-terminally extended form of SS-14, sharing the last 14 AA. The biochemical importance of this region for receptor binding is unknown at this

time.

SS-28 is processed through a divalent cation-sensitive endoprotease which cleaves at the monobasic AA, arginine (Arg). SS-14 is exclusively generated by dibasic cleavage at the Arg-Lys site of the intact precursor with concomitant production of ProSS[1-76] (Brakch *et al.* 1991). Brakch *et al.* (1995) used recombinant vaccinia virus vectors to coexpress each of the candidate prohormone convertases PC_1, PC_2, furin, PACE4 and PC_5 with rat PSS in the constitutive secreting cell line LoVo and in the endocrine corticotroph cell line AtT-20, which exhibits regulated secretion. In LoVo cells PC_1, furin and PACE4 generate SS-14, SS-28 and a mixture of SS-14 and SS-28, respectively, while PC_2 is unable to process PSS in these constitutive cells. In contrast, PC_2 can generate SS-14 in AtT-20 cells. The convertase PC_5 is unable to process PSS in either cell line. Currently therefore it is suggested that PC_2, PC_1 and PACE4 are candidate SS-14 convertases, while PACE4 and furin are candidate SS-28 convertases.

The site of PSS processing is the golgi apparatus in rat neural cells (Lepage-Lezin *et al.* 1991). The two distinct endoproteolytic events, responsible for the release of bioactive fragments SS-14 and SS-28, are performed in the golgi apparatus of both cortical and hypothalamic neurons. PSS is approximately five times larger than the bioactive SS-14 form suggesting that processing of SS-14 occurs in the cell of origin and is then secreted. SS transfected GH_3 pituitary cells, however, show that both PSS and SS-14 are efficiently transported through the secretory pathway and secreted into the culture media (Elgort and Shields 1994). This leads to the conclusion that the propeptide has all the topogenic information necessary for intracellular transport and could possibly function as a novel active peptide in a paracrine fashion.

Somatostatin Metabolism

Degradation of SS by neuronal and glial cells is mediated by an astrocytic metallo-endoprotease which cleaves SS at the Phe^6-Phe^7 and Thr^{10}-Phe^{11} bonds (Lucius and

Mentlein 1991). Astrocytes embrace the neuronal synapses where SS is released and secrete endopeptidases 24.15, 68 kD, and 24.16, 70 kD (Mentlein and Dahms 1994).

SS-14 is also rapidly metabolized in plasma with a half life of less than 2 min in humans (Ho *et al.* 1986; Sheppard *et al.* 1979). In rats metabolism of SS-14 is even faster with a half life of less than 0.5 min. *In vitro*, however, the disappearance of SS-14 in plasma is extended to 1hr (Patel and Wheatley 1983). SS-28 degrades at a rate fourfold slower than SS-14. Due to rapid metabolism, the probability of peripheral SS affecting the CNS is decreased.

SS Receptors

The actions of SS are mediated via membrane-bound receptors. Five distinct G protein-coupled SS receptor (SSTR) subtypes, SSTR1-5, are known (for review see: Reisine and Bell 1995). These receptors are closely related to the opioid receptor family and contain seven transmembrane segments (Reisine 1995). All subtypes are expressed in the CNS. These receptors may exert inhibitory effects in 4 ways: 1) via a receptor that is negatively coupled to adenylate cyclase through an inhibitory G protein (G_i); 2) via a subset of potassium channels that are directly coupled to the SS receptor through a G protein (G_k) leading to the opening of K^+ channels and the hyperpolarization of the neurons, followed by a secondary reduction in intracellular calcium; 3) via a direct action on voltage-dependent calcium channels to block calcium current via G proteins; 4) a distal effect via the receptor on exocytotic secretion (Bissette and Myers 1992).

There are two main classes of receptors. SSTR1/SSTR4 are grouped as somatostatin-1 and SSTR2/SSTR3/SSTR5 compose the somatostatin-2 class of receptors. Presently, no differences in affinity for binding SS-14 versus SS-28 have been consistently shown. Preferential expression of SSTR2 over SSTR1 mRNA in structures such as the dorsomedial and ventromedial nuclei correlates well with earlier demonstrations of preferential binding of the SSTR2 selective ligand MK-678 (Beudet *et al.* 1995). Other

sites in the hypothalamus which contain SSTR1 and SSTR2 are in the same area that immunohistochemistry has shown SS to be, supporting the idea of self modulation and colocalization of different receptor types in the same cell.

SSTR2 exists in two variant forms, SSTR2a and SSTR2b, generated by alternative splicing of the SSTR2 mRNA. SSTR2a has been specifically studied by Dournaud *et al.* (1996) for its localization in the rat brain. Results show the receptor to be associated with both somatodendritic and axonic elements, suggesting its involvement in the transduction of both pre- and postsynaptic effects of SS in the mammalian brain. It is also widespread in the cerebral cortex and limbic structures suggesting this receptor plays a critical role in mediating SS effects on cognition, expression of emotional behavior, learning and memory.

In situ hybridization studies of the SSTR in the pituitary of the rat have suggested specific target hormones for the various receptors (O'Carroll and Krempels 1995). All five SSTR are present in the anterior pituitary. 1-4 are in the intermediate lobe and 2 and 3 are in the neural lobe. Colocalization of SSTR2 in TSH producing cells supports the claim that SS inhibits TSH release. SSTR4 and SSTR5 are more prevalent on GH cells. SSTR3 is found throughout the pituitary which is consistent with findings in other tissues suggesting involvement in functions other than humoral regulation.

Somatostatin Distribution in the CNS

SS has a wide distribution in the brain. For this reason, it is suggested to be a neurotransmitter as well as a neurohormone. SS is found in the hypothalamus, cerebral cortex, brainstem and lymbic system of mammals. Immunohistological and radioimmunoassay techniques have demonstrated high concentrations of SS within interneurons of the cortex and hippocampus, as well as discrete neurons of the hypothalamus and basal forebrain (Johansson *et al.* 1984). SS mRNA has been found in nonpyramidal neurons in the cortical and hippocampal neurons supporting the hypothesis

that SS functions in local circuitry in the forebrain (Fitzpatrick-McElligott *et al.* 1988). In the frontal and parietal cortex, SS is thought to be involved in cognitive processes and memory. A decrease in SS mRNA is seen in memory loss and aging (Dournaud *et al.* 1996). Abnormal levels of SS have been measured in diseases such as Alzhiemer's, schizophrenia, narcolepsy, Cusshings syndrome, Parkinsons disease, manic depression and Huntington's disease.

Hypophysiotropic SS is confined to neurons in the periventricular nucleus (PeN; Epelbaum *et al.* 1977) and arcuate nucleus (Willoughby *et al.* 1989). The PeN projects to the median eminence (ME) pituitary portal vasculature in rats (Merchenthaler *et al.* 1989). This allows SS to function as a hypophysiotropic hormone, controlling the release of anterior pituitary hormones such as GH, thyrotropin stimulating hormone (TSH) (Vale *et al.* 1974), prolactin (Vale *et al.* 1974), and adrenocorticotropic hormone (ACTH) under certain conditions (Voight *et al.* 1977).

Colocalization of SS with other peptides suggests intrahypothalamic regulation of pituitary hormones. SS found in the arcuate nucleus may participate in the regulation of growth hormone-releasing hormone (GHRH) found in the arcuate nucleus but not in the PeN. Double immunohistochemical staining revealed contacts of beta-endorphin or alpha-MSH containing fibres with a majority of somatostatin perikarya in the anterior hypothalamic PeN. This data indicates that hypothalamic SS and proopiomelanocortin (POMC) neurons are interconnected possibly also for the control of GH secretion (Fodor *et al.* 1998). Nerve contacts are also seen between TRH neurons and SS neurons. Approximately 60% of pro-TRH cell bodies were innervated by at least one SS terminal (Liao *et al.* 1992). These nerve contacts are exclusively found in the medial and periventricular subdivisions of the parventricular nucleaus (PVN).

Somatostatin Gene

Complementary DNA (cDNA) sequences encoding for SS were first determined using the mRNA rich Brockmann body of the anglerfish, *Lophius americanus*, (Hobart *et al.* 1980). Distinct cDNAs were found to encode SS-14 and SS-28 encoding [Tyr^7, Gly^{10}]SS-14 in the anglerfish. In the human, a single cDNA with two processing sites for the cleavage of SS-14 and SS-28 peptides has been described (Shen *et al.* 1982) and the gene for SS sequenced (Montminy *et al.* 1984). A distinct gene for SS-28 has not been found in mammals.

The transcriptional unit of the SS gene sequenced from a rat gene library includes two exons of 238 and 367 bp separated by one intron of 621 bp (Montminy *et al.* 1984; Tavianini *et al.* 1984). The human gene also consists of two exons separated by an intron of 877 bp (Shen and Rutter 1984). In order to align the two intron sequences significant gaps must be added.

Somatostatin Gene Expression

The expression of PSS in mammals is found to be tissue specific (Morel *et al.* 1983). For example SS gene expression in the frontal cortex, parietal cortex, and striatum of the rat brain declines with aging (Florio *et al.* 1991) where as an increase is seen in hypothalamic tissue (Leidy *et al.* 1990; Gil-Ad *et al.* 1991). One study, however, has shown SS to decrease with age in the hypothalamus (Martinoli *et al.* 1991). Neurotransmitters also have a role in increasing SS gene transcription. Norepinephrine reduces SS content in the rat hypothalamus by an $\alpha 2$-adrenergic pathway (Gil-Ad *et al.* 1991) where as $\alpha 1$-adrenoreceptor blockade stimulates SS synthesis (Cardinali *et al.* 1994). Factors that elevate cyclic AMP (cAMP) levels such as dopamine D_2 receptor activation, induce SS gene expression. A cAMP response element is present in the SS

gene promoter (Montminy and Bilezikjian 1987) and cAMP induces SS gene transcription (Montminy *et al.* 1986b; Gonzalez and Montminy *et al.* 1989). cAMP has also been found to stimulate transcription of PSS mRNA in the rat diencephalon (Montminy *et al.* 1986a). Peptide hormones that influence SS gene expression will be discussed later

Growth Hormone Feedback Axis

Daily cycle of growth hormone

GH is secreted from the pituitary in an episodic or pulsatile fashion, with the hypothalamus as the main control center. Pulsatile release aids in decreasing the net amount of extracellular GH required to elicit biological responses in target cells (for review see Harvey *et al.* 1995). The amplitude and frequency of pulsatile GH release has been shown be modulated by sleep, stress, exercise and postprandial glucose concentrations. It also varies during growth and sexual maturation, with gender and physiological and pathological conditions (Harvey *et al.* 1995).

GH secretion is sexually dimorphic in the mature rat. High amplitude GH pulses occur in the adult male rat approximately every 3 hours, superimposed on a low or undetectable baseline plasma GH level (Tannenbaum and Martin 1976). Adults female rats have an irregular pattern of low amplitude GH pulses superimposed on an elevated interpulse baseline plasma GH level (Saunders *et al.* 1976). Pulse frequency in humans can range from a maximum measured value of 31.2 pulses/24 hrs in a premature infant (Wright *et al.* 1992) or 32 pulses/24 hrs in a fasting human (Hartman *et al.* 1992) to a minimum measured value of 7.9 pulse/24 hrs in female adults in mid follicular phase (Faria *et al.* 1992) or 3.2 pulses/24 hrs in an obese individual (Veldhuis *et al.* 1991). In all mammals tested so far the maximum pulse frequency is 26.4 pulse/24 hrs in the rat (Bruhn *et al.* 1992) and minimum is 3.9 pulse/24 hrs in the adult female horse (Stewart *et al.* 1993) or 5 year old ram (Morrison *et al.* 1981).

The regulation of this pulsatile release is therefore multifactorial, with hypothalamic stimulators and inhibitors of GH playing a major role. In the rat SS and GHRH peptides in the hypophyseal portal system fluctuate parallel to the decreases and increases, respectively, in GH. When GH is high in serum, SS concentrations are low in the hypophyseal portal blood. SS mRNA oscillates in reverse of SS peptide in the ME and therefore the regulation may be through a transcriptional oscillator, which controls SS at the level of mRNA transcription (Zeitler *et al.* 1991). Murao and coworkers (1995) have shown no correlation between the rise in GH secretion and the rise in SS mRNA in the mouse. No distinct pattern was seen in the amounts of hypothalamic SS measured over a 24 hr period. Whether a similar model of SS and GHRH oscillation applies to other vertebrates is not known.

Short-loop feedback

SS is the major inhibitor of GH secretion and therefore is an integral part of the GH neuroendocrine regulatory system (for review see Harvey *et al.* 1995). GH has the ability to control its own secretion by feedback onto hypothalamic neurons. In 1991, Zeitler *et al.* proposed a model for GH shortloop feedback in the control of GH secretion. GH receptors are colocalized in the hypothalamic SS neurons (Burton *et al.* 1992). Therefore it is possible GH is a direct regulator of SS mRNA production. GH has previously been found to have a direct stimulatory effect on hypothalamic SS synthesis and release (Berelowitz *et al.* 1981a). GH is also seen to increase c-fos, a protooncogene, expression in SS neurons in the arcuate nucleus. When Pellegrini *et al.* (1996) injected GH receptor mRNA antisense into the rat brain, preventing the transcription of GH receptors in the brain, SS expression was seen to decrease in rats. GH, therefore, can feedback directly on SS expression in the brain. The source of the GH may be from the pituitary or the CNS itself.

It is still uncertain whether GH crosses the blood-brain barrier. By injecting

radiolabeled GH into the systemic circulation, no uptake was seen in the brain stem (Pacold *et al.* 1978). Uptake of the radiolabeled GH was seen, however, in the telencephalon, diencephalon, midbrain, pons-medulla and cerebellum (Stern *et al.* 1975). Possible types of entry into the brain include carrier mediated transport (Banks and Kastin 1985) or circumventricular organs such as the median eminence, through retrograde transport from the pituitary (Sato *et al.* 1989), or the choroid plexus (Lai *et al.* 1991). Immunoreactive GH is found in perikarya, nerve fibers and axon terminals throughout the CNS, and are seen to be localized in association with SS and TRH (for review see; Harvey 1993). GH mRNA, however, is only found in the PeV in the hypothalamus along with areas in the telencephalon, thalamus, and cerebellum (Gossard *et al.* 1987; Martinoli *et al.* 1991). Both the presence of GH receptors on SS neurons and immunoreactive GH around SS neurons suggests an important role of pituitary GH in regulating its own secretion. The percentage of brain GH involved is as yet unknown.

Central insulin-like growth factors (IGF) are also implicated in GH short-loop feedback in the hypothalamus. Reduced levels of IGF-I mRNA with hypophysectomy are seen, whereas an i.c.v. injection of GH increases IGF-I mRNA (Wood *et al.* 1991). GH stimulation of IGF-I, therefore, may cause inhibition of GHRH synthesis and release, as well as stimulating SS synthesis and release (Sato and Frohman 1993; Tannenbaum *et al.* 1983).

Long-loop feedback

Many peripheral factors have been implicated in the feedback of GH. IGF-I, synthesised by the liver and other peripheral tissues in response to GH stimulation, has been implicated in the up regulation of SS gene expression (Tannenbaum *et al.* 1983: Berelowitz *et al.* 1981b). It is not known, however, whether the IGF-I levels would increase rapidly enough to cause the observed change in c-fos gene expression. IGF-I can inhibit GH minimally at the pituitary cell level, but it is also known to have positive

interaction with SS. IGF-I can increase somatotroph sensitivity to SS as well as increasing SS receptor binding capacity (Katakami *et al.* 1985). Other factors involved in long-loop feedback include pancreatic hormones, thyroid hormones, adrenal hormones, gonadal hormones, immune factors and metabolic factors (for review see; Harvey 1993).

GHRH and somatostatin hypothalamic interactions

Several lines of evidence suggest that SS regulates GH release in part through central control of hypothalamic GHRH neurons. Colocalization of SS and GHRH in the arcuate nucleus has been demonstrated using immunohistochemistry (McCarthy *et al.* 1992). Colocalized SS and GHRH cells are concentrated rostrocaudally in the ventrolateral portion of the arcuate nucleus. More dorsally and laterally placed cells are rarely colocalized. SS binding studies by McCarthy *et al.* (1992) found SS receptors on GHRH cells, supporting the concept of interactions between SS and GHRH. SS causes a paradoxical elevation of GH when given by an i.c.v. injection. If the GHRH neurons in the arcuate nucleus are destroyed by MSG-treatment, this stimulation of GH ceases to exist (Slama *et al.* 1993). This shows a possible role for SS in the regulation of GHRH.

SS binding sites are associated with a subpopulation of GHRH-containing neurons in the arcuate nucleus. Reisine and Bell (1995) hypothesised that since somatostatin has been shown to inhibit GHRH neurons, it is possible that SSTR2 may be involved in the control of GHRH neuronal activity by somatostatin. Guo *et al.* (1996) show that both SSTR1 and SSTR2 in the brain are under the regulatory influence of pituitary hormones. The effects of hypophysectomy and hypophysectomy with GH replacement in rats illustrate how the two receptor levels decreased in hypophysectomized rats. An injection of GH increases the supressed levels of SSTR mRNA, but not up to the levels of intact animals. The decrease of mRNA in the arcuate nucleus has been shown to be directly associated with cells both containing and expressing GHRH. In the arcuate nucleus, SS may directly

modulate GHRH release into the hypophyseal portal blood, and thereby influence GH secretion, through interaction with both SSTR1 and SSTR2 receptor subtypes (Tannenbaum *et al.* 1998)

Factors Affecting Brain Somatostatin Expression and Secretion

There are many factors known to modulate SS gene expression and secretion in the the brain of mammals (for review see; Harvey 1993). The following will review only the factors manipulated during experiments in this thesis; sex steroids and dopamine.

SS and Sex Steroids

Possible sexual differences in the feedback regulation of GH secretion were investigated by measuring immunoreactive SS in the ME of male and female rats bearing the MtTW15 tumour, which secretes high amounts of GH and prolactin (Maiter *et al.* 1990). SS immunoreactivity was increased by 40% in male rats, where as no change was observed in the female rats. Female rats used in this experiment were proestrous, when SS peptide content is at the lowest in the reproductive cycle (Knuth *et al.* 1983). Through push-pull cannulation in the ME, Estupina and colleagues (1996) found that whatever the stage of the estrous cycle, SS release from the ME is not uniform and exhibits irregular peaks. Mean SS release levels, however, were subjected to gonadal influence, whereas the occurrence of the SS peaks seems to be estrogen-independent.

In the female rat, ovariectomy provokes a decrease in the hypothalamic SS content accompanied in most cases (Baldino *et al.* 1988; Werner *et al.* 1988) but not always (Hasegawa *et al.* 1992) by a decrease in SS mRNA levels. Estradiol has also been shown to significantly increase the number and binding density of ^{125}I-SS-labeled cells in the arcuate nucleus of ovariectomized rats. ^{125}I-SS binding was also significantly higher in proestrous as compared to diestrus, diestrus II and estrus (Slama *et al.* 1992). Therefore,

estradiol is a positive regulator of SS binding in the arcuate nucleus of the hypothalamus. Variation can be seen, however, between SSTR5 and SSTR2A receptor subtypes in normal rat pituitaries (Kimura *et al.* 1998). There is a sex-dependent difference, males higher than females, and as E_2 up-regulates the expression of SSTR2 isoforms, it down-regulates the expression of SSTR5, suggesting roles for these subtypes in the control of pituitary functions. Control of SS by E_2 therefore can exist at the hypothalamic or pituitary level.

In the ram hypothalamus, androgen receptors have been colocalized with SS in the preoptic area and mediobasal hypothalamus, with little or no immunoreactivity for androgen receptors in GnRH or dopaminergic neurons of the preoptic area and hypothalamus (Herbison *et al.* 1996). This could indicate sites of SS-androgen interaction which may in turn have indirect effects on GnRH and dopaminergic neurons. These experiments were performed on rams in the non-breeding season.

Due to a lower baseline of GH secretion found in male rats, SS was hypothesized to be more abundant in males. SS mRNA synthesis is indeed higher in the adult male rat compared to the female rat. A large increase in SS mRNA is also observed in testosterone injected adult females, suggesting that testosterone has a stimulatory effect on SS mRNA synthesis. In both neonatal and adult rats, sex steroids have significant effects on the synthetic capacity of SS neurons in the PeN and PVN, as seen with a decrease in SS in castrated male neonatal rats (Chowen *et al.* 1996). Non-androgenic testicular factors are also involved in the regulation of SS. Knocking out the androgen producing Leydig cells of intact animals and comparing SS mRNA levels to that of castrated animals, shows higher levels of SS in castrated rats (Lago *et al.* 1996). Inhibin secretion is elevated with no Leydig cells present and therefore may be the non-androgen factor inhibiting synthesis of SS. In castrated rats, an increase in inhibin is not seen due to the absence of non-Leydig testicular cells which produce inhibin. Testosterone, therefore, has a stimulatory effect on SS mRNA with a negative influence on SS by non-androgenic factors such as inhibin.

Dopamine

Dopamine (DA) is a catecholamine found in the central nervous system which can act as a neurotransmitter or neurohormone. DA can have biological actions through 5 different families of receptors; D_1-D_5 (Sidhu *et al.* 1998). Only D_1 and D_2 receptors will be discussed for comparison to known fish D_1 and D_2 receptors. D_1 receptors have a high binding affinity for 1-phenyl benzepines (eg SKF 38393) whereas D_2 receptors are selective for substituted bezamides and butyrophenones (eg (-) sulpiride and spirerone; Nizinik, 1987). The two receptor families also have unique signal transduction pathways. D_1 receptors are positively coupled to the cAMP producing enzyme adenylate cyclase via a stimulatory G protein (G_s) (Battaglia *et al.* 1986b; Monsma *et al.* 1990). SKF 38393 is a D_1 agonist which increases cAMP production (Battaglia *et al.* 1986a,b). D_2 receptors are negatively coupled with adenylate cyclase through an inhibitory G protein (G_i) (Enjalbert and Bockaert 1983; Elazar *et al.* 1989).

The effects of DA on GH in mammals are controversial. Stimulatory actions of L-DOPA, DA and the DA agonist apomorphine on GH release have been reported in the rat (Sonntag *et al.* 1982; Meister and Hulting 1987), dog (Casanueva *et al.* 1981), baboon (Steiner *et al.* 1978), human (Vasavan Nair *et al.* 1986; Delitala *et al.* 1987; Lal *et al.* 1991), and non human primates (Chambers and Brown 1976). Suppression of plasma GH level is seen when the catecholaminergic pathways in the brain are disrupted by reserpine (Eden *et al.* 1979) and 6-hydroxydopamine (Willoughby and Day 1981). No effect on GH due to dopamine was seen in the rat (Bluet-Pajot *et al.* 1980) and human (Jordan *et al.* 1986) but these can be attributed to different ways of drug administration, pathological conditions of the animal model and interspecies variations of GH responses. They also may result, however, from the complexity of DA and GH interactions.

DA can modulate GH secretion at two levels. Indirect actions can be seen at the hypothalamic or pituitary level and direct actions may occur at the level of somatostrophs in

the pituitary. SS is one of the hypothalamic peptides implicated in the indirect effects of DA on GH release. In the rat, DA stimulates SS release *in vitro* from hypothalamic synaptosomes (Wakabayashi *et al.* 1977), perifused hypothalamic slices (Terry *et al.* 1980; Kitajima *et al.* 1989) and hypothalamic cells in primary culture (Richardson *et al.* 1983). *In vivo* experiments with i.c.v. injection of L-DOPA also increases SS levels in the hypophyseal portal blood (Chihara *et al.* 1979) and depletes SS content in the hypothalamus (Torres *et al.* 1982; Cheung *et al.* 1997). These actions of DA are mediated through D_2 receptors (Lewis *et al.* 1986). *In vitro* experiments with bovine hypothalamic sections show that SS secretion increases with D_1 receptor stimulation and also mediates the inhibitory effects of activation of D_1 receptors on the release of GHRH (West *et al.* 1997). Indirect actions of DA on SS in the hypothalamus therefore point to the inhibition of GH by DA. Increasing SS secretion from the hypothalamus would cause a decrease in GH secretion from the pituitary.

Somatostatin in Teleosts

Forms of Somatostatin Found in Lower Vertebrates

Teleosts are unique in the evolution of SS due to the presence of two SS preprohormones. The second precursor for a unique large form of SS (preproSS II) was identified in cDNA from rainbow trout (*Oncorhychus mykiss* ; Moore *et al.* 1995), flounder (*Platichthys flesus*), sculpin (*Cottus scorpius*; Conlon *et al.* 1987), coho salmon (*Oncorhynchus kisutch*; Plietskaya *et al.* 1986) and eel (*Anguilla anguilla*; Conlon *et al.* 1988). The catfish, *Ictalurus punctatus*, has a unique SS-22 peptide (Minth 1982) and lamprey species have various unique tissue specific forms processed from PSS II (for review see; Wei *et al.* 1997). Bowfin, *Amia calva*, have only a single precursor identified so far for SS-26 and SS-14 peptides (Wang *et al.* 1993).

SS-14 is conserved from lower vertebrates through mammals (for review see; Conlon 1990). In addition, recent investigations have revealed four molecular variants of SS-14: [Ser^5]SS-14 in islet cells of ratfish, *Hydrolagus colliei* (Conlon 1990), [Pro^2]SS-14 in pituitaries of Russian sturgeon, *Acipenser gueldenstaedti* (Nishi *et al.*. 1995), [Ser^{12}]SS-14 in the pancreas of sea lamprey, *Petromyzon marinus* (Andrews *et al.*. 1988) and [Pro^2, Met^{13}]SS-14 from the brain of frogs, *Rana ridibunda* (Vaudry *et al.* 1992).

Distribution of Somatostatin Immunoreactivity in the Brain

Immunocytochemical studies in goldfish as well as a wide variety of other teleosts have shown that cell bodies and nerve fibers containing immunoreactive somatostatin (SS-IR) are widely distributed throughout the central nervous system (Dubois *et al.* 1979; Grau *et al.* 1985; Kah *et al.* 1982; Olivereau *et al.* 1984 a, 1984b; Vigh-Teichman *et al.* 1983). The regional distribution does not vary greatly between species. Immunoreactive cell bodies are present in several nuclei in the preoptic and periventricular regions, including the nucleus preopticus periventricularis (NPP), nucleus anterioris periventricularis (NAPv), and the nucleus lateral tuberis (NLT) A small number of immunoreactive neurons can also be found in the nucleus anterior tuberis close to the recessus lateralis. Fibers from nuclei in the forebrain form a dense plexus of SS-IR fibers in the periventricular region of the hypothalamus with some fibers from this plexus extending through the basal hypothalamus and the infundibulum to terminate within the pituitary gland.

In cyprinid species such as the goldfish (Kah *et al.* 1982; Olivereau *et al.* 1984b) SS-IR fibers occur in the proximal pars distalis (PPD), scattered among the GH secreting cells, somatotrophs. Nerve fibers containing SS are also observed in the caudal neurohypophysis and amongst MSH cells of the pars intermedia (Olivereau *et al.* 1984b). In other species such as the eel (*Anguilla anguilla*; Olivereau *et al.* 1984a) and various salmonids (Dubois *et al.* 1979; Olivereau *et al.* 1984a), SS-IR fivers terminate on the basal

lamina of the neurohypophyseal tissue within the PPD. The tilapia (*Oreochromis mossambicus*) also has SS-IR fibers found in neurohypophyseal tissue within the rostral pars distalis (Grau *et al.* 1985). The site of synthesis of SS peptides is not known in teleosts. SS-IR is present in the pituitary but is it produced there or only transported there through nerve fibers originating in higher brain centers? Immunoreative studies, to date, in teleosts have also not been able to distinguish between the distribution of the different forms of SS known. Studies of gene expression will help to answer these unknown questions.

Somatostatin and Growth Hormone in Teleosts

In goldfish, SS peptides have been tested for their effects on pituitary GH secretion and it has been confirmed that SS-14 is inhibitory (Peter and Marchant 1995). Investigations by Cook and Peter (1984) linked SS-14 injection to transient decreases in GH plasma levels. *In vitro* studies on the actions of mammalian SS-14 and -28, channel catfish SS-22 (Marchant *et al.* 1987) and salmon SS-25 (Marchant and Peter 1989) on GH release from perifused goldfish pituitary fragments revealed that SS-14 and -28 are equipotent; however, salmon SS-25 and catfish SS-22 had no effects on GH release. A SS-28 peptide has recently been sequenced from the intestinal tract of goldfish (Uesaka *et al.* 1995). This peptide sequence contains [Try^{7},Gly^{10}]SS-14 at its C-terminus. The action of this peptide on GH release has not been investigated.

In goldfish, GH secretion has large seasonal variations; the highest serum GH concentrations occurring during pre- and post- spawning periods of April-May; the lowest concentrations being in November (Marchant and Peter 1986). The rate of body growth in males and females increases in response to increases in serum GH. The goldfish reproduces on a seasonal cycle with photoperiod and temperature affecting gonadal growth

and maturation (Hontela and Stacey 1990). Therefore in a temperate climate, gonadal development/recrudescence usually occurs in late fall and winter, spawning in the spring and the gonads remain regressed during the summer and early fall. The sexual state of the fish is indicated by the gonadosomatic index (GSI; Kagawa *et al.* 1983). The maximal GSI is observed around just before spawning when both GH and gonadotropin hormone (GTH-II) are at their maximum serum concentration. GH levels remain high for about a month post-spawning and then drop. The relationship between SS peptide levels in the brain and seasonal GH changes in the plasma were investigated by Marchant *et al.* (1988) and an inverse relationship to serum GH was found. No differences were found between male and females in serum GH levels.

GH secretion, in teleosts, is stimulated by gonadotropin-releasing hormone (GnRH), dopamine (DA), growth hormone releasing-hormone (GHRH), neuropeptide Y (NPY), thyrotropin-releasing hormone (TRH), bombesin (BBS), cholecystokinin (CCK; Peng and Peter 1997) and pituitary adenylate cyclase-activating polypeptide (PACAP; Wong *et al.* 1998). As in mammals, modulation between GH stimulating factors and SS may be present. No studies have been conducted to discover the regulators of seasonal variations in brain with respect to the main GH inhibitor, SS.

SS is suggested to inhibit GH release and not transcription. In tilapia, SS had no clear effect on GH transcription levels, but reduced GH release by as much as 90% (Melamed *et al.* 1996). The lack of effect of SS on GH steady state mRNA is similar to that seen in rainbow trout in which GH mRNA levels were unaltered in pituitaries exposed to 25nM SS for 18 hours (Yada and Hirano 1992). GH transcription is also unaffected by SS in mammals (Namba *et al.* 1989).

Sex Steroid Interactions in Teleosts

Elevation of GH during sexual maturation has been reported in the whitesucker

(Stacey *et al.* 1984), rainbow trout (Sumpter *et al.* 1991), goldfish (Trudeau *et al.* 1992), Atlantic salmon (Bjornsson *et al.* 1994), and chum salmon (Kakizawa *et al.* 1995). Plasma sex steroid levels change on a seasonal basis in response to GTH-II secretion and may feedback in the regulation of GH secretion though out the year. In females, plasma levels of E_2 are low from December to February, but rapidly increases in March and remains at high levels during ovulation in April (Kagawa *et al.* 1983). The concentration of T in plasma is higher than E_2 between January and post-spawning. In males, the plasma androgen levels slowly increase as the testicular development increases (Kobayashi *et al.* 1986). An interesting characteristic of testosterone is that is can be aromatized into estradiol through the enzyme aromatase (Nagahama 1994). The administration of gonadal steroid has been shown to increase GH serum levels in goldfish (Trudeau *et al.* 1992), a tilapia hybrid (Melamed *et al.* 1995), and rainbow trout (Holloway and Leatherland 1997). More specifically, E_2 has been identified to stimulate GH secretion in female goldfish, but not to affect steady state GH mRNA levels in the pituitary (Zou *et al.* 1997). The effects of 17β-estradiol treatment on plasma SS were tested *in vivo* and *in vitro* on sexually immature rainbow trout (Holloway *et al.* 1998). Steroid implantation for two weeks significantly elevated GH plasma levels while significantly lowering SS serum levels relative to control. Pituitary fragments taken from E2-primed fish showed no inhibition of GH secretion when challenged with SS-14 (Holloway *et al.* 1998). Sex steroids, therefore, have been shown to have an effect on GH secretion but may act at the level of both production of the SS peptide as well as SSTR regulation.

E_2 receptors (ER) are nuclear receptors that act as ligand-dependent transcription factors on the expression of certain target genes. The rainbow trout estrogen receptor cDNA sequence is known (Pakdel *et al.* 1990) and *in situ* hybridization (Salbert *et al.* 1991; Salbert *et al.* 1993) and immunohistochemistry (Anglade *et al.* 1994; Navas *et al.* 1995) have been used to localize ER in the brain of trout. Results show ER in teleosts are mainly expressed in the preoptic area and the mediobasal hypothalamus, although a smaller

population of ER expressing cells is consistently observed in an area of the ventral telencephalon. It is also known that GnRH neurons in the trout do not express ER (Navas *et al.* 1995) but ER are found to be colocalized with tyrosine hydroxylase immunoreactive cells, ventral to the large extensions of the preoptic recess indicating that this DA producing region is a major target for estradiol feedback (Linard *et al.* 1996).

Intracellular Mechanisms for Somatostatin Inhibition of GH

In goldfish intracellular signal transduction mechanisms mediating the effects of two stimulatory regulators of GH release, GnRH and DA, have been studied (reviewed in Chang *et al.* 1994, 1996). GnRH acts via GnRH receptors to stimulate GH release. This stimulation is mediated by activation of protein kinase C (PKC) and extracellular calcium entry through high voltage-activated, voltage-sensitive calcium channels (VSCC). In contrast, dopamine D_1 action on GH release is mediated primarily by cyclic (c)AMP-dependent mechanisms and PKA but D_1 also utilizes VSCC. This allows for the additive effect seen on GH secretion when the two ligands are used simultaneously. D_1 receptor activation of cAMP-dependent pathways also leads to a secondary increase in calcium entry through VSCC. In tilapia the same D_1 pathway is also seen to increase GH gene transcription (Melamed *et al.* 1996), however, exposure of tilapia pituitary cells to GnRH increases GH release without increasing GH gene expression (Mahmoud *et al.* 1996)

A recent study has been done to elucidate the intracellular mechanisms mediating SS inhibition of stimulated GH release from dispersed pituitary cells (Kwong and Chang 1997). SS is seen to act on D_1 and GnRH-stimulated GH release at sites distal to cAMP production and PKC activation, respectively. SS also exerts its effect at sites distal to calcium mobilization. It is also possible that there is an inhibitory action at the level of extracellular calcium entry through VSCC.

Objectives of Study

The purpose of this study was to elucidate the factors affecting SS mRNA expression and accumulation in the goldfish brain. In the mammalian system, SS mRNA expression has been well studied with regards to the regulation of GH release. GHRH and SS are the main regulators of GH release in mammals, whereas there are many stimulators of GH secretion in the goldfish. GnRH, as well as, DA are major stimulators of GH secretion. GRF and DA are more potent in stimulating GH secretion in sexually regressed fish, whereas, GnRH, NPY, and TRH are more potent in mature (prespawning) fish. GnRH and DA, through D_1 receptors, are considered the most potent stimulators *in vitro*.

SS is the main inhibitor of GH secretion. Serotonin (5-HT) and norepinephrine (NE) also have inhibitory actions on GH release *in vitro* in the goldfish (Peter *et al.* 1990; Somoza and Peter, 1991: Wong, 1993 a&b), and intraperitoneal injection of NE also suppresses serum GH levels in the goldfish (Chang *et al.* 1985). However, the concentrations of 5-HT are very low and NE is not detectable in the goldfish pituitary (Sloley *et al.* 1991). The physiological importance of these factors in regulation of GH secretion has yet to be determined.

At the beginning of this study, there were two known cDNA sequences for SS cloned from the brain of goldfish; one sequence encoding the mature peptide sequence for mammalian SS-14 and a second encoding the mature peptide for a unique goldfish SS-28. The presence of two mRNA sequences for SS suggests the possibility for differential regulation of gene expression for the two hormones. The first objective of this study was to confirm the sequence of SS-28 through library screening, resulting in the discovery of a third form of SS mRNA from the cDNA library of the goldfish brain. The inhibitory effect of this new form of SS-14 on GH was tested through *in vitro* perifusion of goldfish pituitary fragments and RIA of GH secretion. Distribution studies were done through Northern and Southern analysis of six defined brain regions, the pituitary and various body

regions. The genomic size of the three mRNA was confirmed and genomic SS-28 was sequenced.

Unlike the rat model, daily cycles in GH secretion have not been identified in the goldfish. There is, however, a very strong seasonal cycle in GH secretion (Marchant and Peter 1986). My second objective was to identify any seasonal variations in SS mRNA expression and accumulation in the forebrain. This experiment demonstrated distinct patterns of SS gene expression for each mRNA. An experiment testing the effects of sex steroids *in vivo* on the two forms of SS-14 mRNA expression was performed.

The third objective of this study was to start the investigation of intrahypothalamic control of GH secretion. DA and GnRH were the two GH stimulators used in this *in vivo* study. The effects of both D_1 and D_2-receptor agonists and sGnRH on SS mRNA expression were studied.

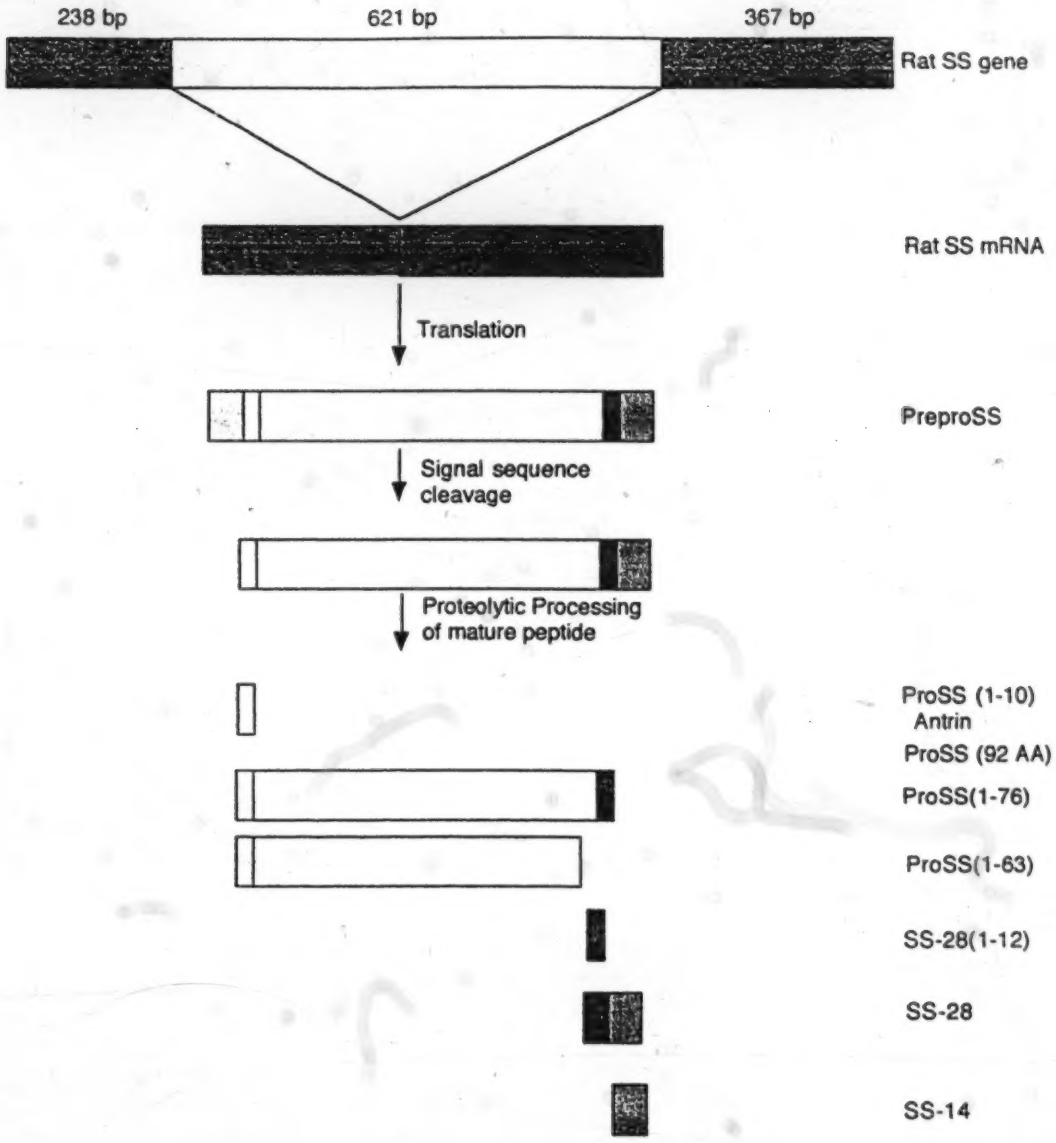

Figure 1-1: Rat SS gene, messenger RNA and mature peptide products.

CHAPTER 2

Material and Methods

Molecular Cloning of cDNA Encoding PSS-I[2]

Screening of cDNA Library

A 426 bp DNA probe was prepared by polymerase chain reaction (PCR) with primers specific for the known sequence PSS-II (see Table 2-1). The template was PSS-II previously inserted into a plasmid and sequenced. Cycles for PCR included 5 min denaturing at 95°C, then 30 cycles of 1 min denaturation, 94°C, 1 min annealing, 55°C, 1 min extension, 73°C, with an end cycle of 10 min at 73°C for elongation of the fragment.

A goldfish brain cDNA library (kindly provided by Dr. H.R. Habibi, University of Calgary, Calgary, AB, Canada) was constructed using the ZAP-cDNA synthesis kit, including Gigapack II Gold packaging extract (Stratagene, La Jolla, California, USA). The library was amplified once to a titer of $6X10^9$ pfu/ml before being transferred to Nybond-N+ disc membranes (Amersham LIFE SCIENCE, Oakville, ON, Canada) at a density of $2X10^4$ plaques/filter according to the manufacturer's protocol. The probe labeling, hybridization and signal detection were performed using enhanced chemiluminescence (ECL) direct nucleic acid labeling and detection systems (Amersham)(Whitehead *et al.*, 1983). Stringency washing was carried out with 6 M urea, 0.4% SDS and 0.5X Sodium chloride/Sodium citrate (SSC) for 15 min twice at 42°C, and 2XSSC for 10min twice at room temperature. A total of $2.4X10^5$ clones were screened, out of which six positives were picked and subjected to the second colony hybridization with the same probe and hybridization conditions.

Subcloning and Sequencing

Phagemids pBluescript SK(-) containing a cDNA insert were excised from the phage DNA of positive plaques using *in vivo* excision according to the instruction of the ZAP-cDNA synthesis kit. Three clones with a cDNA insert of desired size (approximately 750 bp) were sequenced on an Applied BioSystems automated sequencer (373A), by the Biochemistry department at the University of Alberta, according to the manufacturer's protocol. Sequencing was carried out on both strands using T_7 and T_3 sequencing primers that flank the insert cDNA. DNA sequences were analyzed using DNA Strider and Clustal W software.

Isolation and Sequencing of Gene for PSS-II

Sexually regressed female and male goldfish (30-40 g body weight) were purchased from Grassyfork fisheries (Martinsville, Indiana). The fish were maintained in 300-liter flow through aquaria at 17°C under a 16-hr light and 8-hr dark photoperiod and were fed twice daily, to excess, with commercially prepared Unifeed Nu-way trout ration (United feeds, Calgary, Alberta). Goldfish were anesthetized with 0.05% tricaine methanesulfonate (MS 222, Syndel, Vancouver, BC) before liver tissue collection.

Genomic DNA was extracted from the goldfish liver using the Trizol DNA extraction method (GIBCO/BRL). PCR amplification was performed using 1 µl of genomic DNA as template and primers F1 and R1 designed from the 5' and 3' ends of the known PSS-II cDNA sequence. A control tube, with primers, TAQ, and no genomic template, was made to ensure there was no contamination in the PCR reaction. The PCR cycles were the same as that used for the synthesis of PSS-II probe described above. PCR products were separated by agarose gel electrophoresis. After electrophoresis on a 1.5% agarose gel, two identical bands were then isolated using Geneclean and subcloned into the

pGEM-T vector (Promega, Madison, WI, USA). Transformation was performed using XL-1 blue cells and positive colonies, identified through PCR with primers F1 and R1, were chosen for mini plasmid prep and sequencing. DNA sequence analyses was carried out on an Applied BioSystems automated sequencer (Perkin-Elmer, Mississauga, ON, Canada) according to the manufacturer's protocol in the department of Biochemistry at the University of Alberta. Both strands of cloned DNA were sequenced in opposite directions using T_7 and M_{13} reverse sequencing primers that flank the inserted DNA. Primers were then designed from the sequence ends to sequence the middle section of the DNA more accurately (Table 1). DNA sequences were analyzed using DNA Strider.

Table 2-1. Primers used for sequencing the complete gene sequence encoding preprosomatostatin-II from the liver of goldfish. See figure 3-4 for location of primers in sequence.

Primers	Sequences
R1	5'CAAGCGAGGGCCTCAGCAGG3'
F1	5'CGAATCACAGCTACAAAGAGTC3'
R2	5'TCCATTAACTCAGCGTTCCT3'
F2	5'CCCCTGGATTCTAGTTTTCC3'
F3	5'GGTGAACTATTGCTCAAACA3'
R3	5'TCTCTTTGTAAAGTCCTGAG3'

Differential Expression of the Three Somatostatin Genes

Preparation of Total RNA

Sexually regressed female and male goldfish (30-40 g body weight) were purchased from Grassyfork fisheries (Martinsville, Indiana). The fish were maintained in 300-liter flow through aquaria at 17°C under a photoperiod of Edmonton, AB, Canada,and were fed twice daily, to excess, with commercially prepared Unifeed Nu-way trout ration

(United feeds, Calgary, Alberta). Goldfish were anesthetized with 0.05% MS 222 before brain collection.

Total RNA was extracted from five discrete brain areas: olfactory bulbs and tracts; telencephalon, including optic nerve and preoptic region; hypothalamus; optic tectum-thalamus, including anterior part of cerebellum; and posterior brain (cerebellum-medulla-spinal cord) (see Figure 2-1). Total RNA from the pituitary gland was also extracted for northern blot analysis. A single fish was used for all areas except the pituitary and olfactory bulbs in which a pool of 5 pituitaries and pairs of olfactory bulbs were required to have a detectable amount of RNA in each sample by Northern blot analysis. Six peripheral body tissues were also sampled: muscle, liver, kidney, gonad, and gastrointestinal tract (GI). Approximately 100 mg of tissue was taken from each region. Trizol reagent, based on the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomzynski and Sacchi 1987), was used for RNA extraction. Final RNA concentrations were determined by optical density (OD) readings at 260nm and integrity of the RNA was verified by ensuring the ratio of OD 260/280 was between 1.8 and 2.0. Samples were stored at -70°C.

Preparation of messenger RNA from Pituitary Cells

Pituitary cells were prepared as described previously by Chang *et al.* (1990). $1x10^5$ $-1x10^{10}$ cells were spun briefly and total RNA was extracted as described above. Purification of mRNA was then performed on pituitary cell total RNA using the PolyAtract mRNA Isolation System (Promega, Madison, WI, USA). In brief, total RNA, heated to 65°C for 10 min, was hybridized with biotin-oligo (dT) for 10 min at room temperature. Streptavidin MagneSphere Particles were added and incubated at room temperature for 10 min. Samples are magnitized and washed 4 times with 0.1X SSC and mRNA was eluted twice with RNase-Free water.

Probe Synthesis for Southern and Northern Analysis

Probes for PSS-I^1, PSS-I^2 and PSS-II mRNA were designed to span from the peptide-encoding region to the C terminus prior to the poly (A) tail of the known cDNA sequence. Probes were synthesized by PCR using specific primers designed from the known cDNA sequences (see Table 2-2) and pGEM-T plasmid DNA containing cDNA inserts, for the desired probe, as template. PSS-I^1 probe was amplified through PCR with 5 min at 94°C, 30 cycles of 1 min 94°C, 1 min 50°C, 1 min 73°C, and one cycle of 10 min at 73°C for elongation. The template was plasmid containing the PSS-I^1 insert and the amplified fragment is 346 bp. PSS-I^2 probe is 533 bp in length with the PCR annealing temperature at 55°C and the remaining cycles the same as PSS-I^1. Probe for PSS-II was synthesised as described above for library screening. Plasmid with an α-tubulin insert was a kind gift from H. Habibi at the University of Calgary. The plasmid DNA was digested with Eco R1 at 37°C from 3 hrs to overnight. All four probes were separated by electrophoresis and purified by GeneClean.

Table 2-2. Primers used in PCR for synthesis of the three PSS probes. F-forward primers; R-reverse primers. Template for reactions were known plasmid vector inserts. See figures 3-1, and Appendix 1a&b for location of primers on sequences.

Primers	Sequences
PSS-I^1 F	5'GCGTATCCAGTGCGCACTGGC3'
PSS-I^1 R	5'GTGAAAGTTTTCCAGAAGAA3'
PSS-I^2 F	5'GGAGCTACAAG ACTTCAAC3'
PSS-I^2 R	5'CTGTGTCAGAGTAAGTCCACG3'
PSS-II F	5'CGAATCACAGCTACAAAGAGTC3'
PSS-II R	5'CAAGCGAGGGCCTGAGCAGG3'

RT-PCR Assay and Southern Blot Analysis

Four µg of total RNA, of each brain region and body tissue mentioned previously, were reverse transcribed into cDNA with oligo dT-adaptor primer using the SuperScript Preamplification System for first strand cDNA synthesis (GIBCO/BRL). The presence of cDNA after first stand synthesis was tested by PCR of each sample with primers specific for β-actin (Peyon *et al.* 1998). Four µg mRNA was used for pituitary samples and cDNA integrity was tested by PCR using a cholycystokinin (CCK) specific primer set (Peyon *et al.* 1998). RT-PCR reactions of 25 cycles were then performed on each of the 5 brain areas and pituitary with primer sets; PSS-I^1 F and R; PSS-I^2 F and R; and PSS-II F and R. Denaturation was at 94°C for 1 min, annealing temperature was at 51°C for 1 min and 1 min at 73°C for extension. A final extension of 10 min at 73°C was done. PCR amplification was done in the presence of a PCR buffer, Thermus aquaticus (*Taq*) DNA polymerase and primer control. 20 µl of each sample were electrophoresed through a 1% agarose gel. The gel was then incubated twice with denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 20 min each and twice with neutralization buffer (1.5 M NaCl, 0.5 M Tris HCl) for 20 min each. The DNA was then transferred onto a Nybond-N membrane (Amersham LIFE SCIENCE) by capillary transfer with 20X SSC buffer, followed by baking at 80°C for 2 hours. Southern analysis was then performed using ECL direct nucleic acid labeling and detection systems (Amersham LIFESCIENCE). cDNA probes, as described above, were used for PSS-I^1, PSS-I^2 and PSS-II mRNA detection.

Northern Blot Analysis

For each brain region and the pituitary approximately 10 µg of total RNA in 6 µl of water were added to a denaturing solution of 12.5 µl formamide, 2.5 µl 10x 3-(N-morpholino)-propamesulfonic acid (MOPS), and 4 µl formaldehyde solution (37%). The

samples were then heated at 65°C for 10 min and loaded onto a 1.5% agarose gel. The gel and running buffer contained water, formaldehyde, and 10xMOPS at ratios of 80:8:10 respectively. All samples for the same experiment were loaded onto the same gel which was run at 70 V for 3-5 hours. The gel was then washed in RNase-free water until transfer apparatus was set up. RNA was then transferred from the gel to Nybond-N membrane using 20x SSC transfer buffer. All solutions used were RNAse free. Probes were synthesized for PSS-I[1], PSS-I[2] and PSS-II and labeled with ^{32}P dCTP using T7QuickPrime Kit (Pharmacia Biotech, Baie d'Urfe, QC, Canada). Church and Gilbert (1984) hybridization method was used. In brief, membranes were prehybridized in 15 ml hybridization solution (0.5M $NaHPO_4$, 7% SDS, 1mM EDTA, 1% BSA) for one hour. The hybridization solution was then changed and 16 µl of probe was added. After hybridization overnight at 65°C, the membranes were washed with Churches wash solution (0.04 M $NaHPO_4$, 1 mM EDTA, 1% SDS) and exposed to a phosphoimaging screen for 3 days. The same membranes were rehybridized with α-tubulin probe to control for total RNA loaded in each well of the gel. Membranes were hybridized with labeled α-tubulin probe overnight at 65°C and exposed to the phosphoimaging screen for one day. Between hybridizations with PSS probe and α-tubulin probe the membranes were stripped for two hours, to remove the previously bound probe, with stripping solution (5 mM Tris-HCL, pH 8.0, 2 mM EDTA, 0.1x Denhardt solution) at 65°C and allowed to dry at room temperature.

Functional Analysis of [Pro2]SS-14 by *in vitro* Perifusion of Pituitary Fragments.

Perifusion of Pituitary Fragments

In vitro experiments were conducted using a perifusion system for goldfish pituitary fragments as described previously (Marchant *et al.* 1989a). In brief, goldfish

pituitary fragments (≈0.2 mm^3) from 30 pituitaries were divided over 8 columns giving an average of 3.75 pituitaries per column. The fragments were perifused overnight (15-18 hr), with M199 (GIBCO/BRL) containing 56 µg/µl Nystatin, at a flow rate of 5 ml/hr (Sigma). The perifusion medium was switched to Hank's balanced salt solution containing 25 mM HEPES and 0.1% BSA (Sigma), and the flow rate increased to 15 ml/hr. After another 2 hours of perifusion, 5 min fraction collection began.

First the dose response of [Pro^2]SS-14 was tested by administering 2 min pulses of increasing doses of [Pro^2]SS-14 from 0.01 nM to 100 nM concentration with 30 min between each pulse. To test the interaction of [Pro^2]SS-14 peptide with GH secretagogues, 2 min pulses of DA D_1 receptor agonist, SKF 38393, at 1 µM and 5 min pulses of sGnRH at 100 nM were administered before, during, and after the one hour continuous perifusion with [Pro^2]SS-14. The [Pro^2]SS-14 was a gift kindly given to us by Professor H. Kawauchi (Kitasato University, Sanriku, Iwate, Japan). The fractions were collected and stored at -25 °C until their hormone contents were assayed with a carp GH radioimmunoassay (RIA) which has been previously validated for the measurement of goldfish GH (Marchant *et al.* 1989b).

Data Analysis

Hormone data was transformed into "% pretreatment" as defined previously (Wong *et al.* 1992). An average of the first six fractions of a perifusion column is taken and all subsequent values in the same column are measured as a precent of this average, (% pretreatment). This transformation allows pooling of data from separate columns of the same experiment. Post-pulse fractions with hormone contents greater than or less than the mean plus one standard error of mean (SEM) of the basal hormone level three fractions before the pulse were included as a part of the hormone response. GH responses were quantified by calculating the net change in GH release (i.e., area under the curve) after a

particular drug treatment. GH responses were compared using the students *t*-test with $p<0.05$ showing a significant difference. A dose-response curve for [Pro^2]SS-14 and the corresponding values for half maximal effective dose (ED_{50}) were determined by ALLFIT program (DeLean *et al.* 1978).

Regulation of Somatostatin Gene Expression

Reagents and Test Substances

sGnRH was purchased from Peninsula Laboratories Inc. (Belmont, CA). SKF 38393 (1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride), and SCH 23390 (7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrhydro-1H-3-benzazepine hydrochloride), were purchased from Research Biochemicals Inc (Wayland, MA). LY 171,555 ((-)-Quinpirole hydrochloride) was purchased from TOCRIS (Ballwin, MO, USA). Pimozide was a gift from Janssen Pharmaceutica (Beerse, Belgium). Apomorphine ((+/-)apomorphine HCL) was puchased from Sigma Chemical Company (St. Louis, Missouri).

sGnRH was dissolved in 0.1 M acetic acid and then diluted in 0.6% NaCl saline for a final dose of 0.01 µg/g body weight of fish. LY 171,555 was dissolved in saline to give a dose of 2 ug/g body weight of fish. Apomorphine, SKF 38393, SCH 23390, and Pimozide were first dissolved in dimethyl sulfoxide (DMSO), and diluted in propylene glycol for a final dose of 40 µg/g body weight. All injections were given at 5 µl/g of body weight. Final concentration of DMSO was less than 0.1%. DMSO up to 0.1% concentration does not alter basal GH and GTH-II release from the pituitary cells of goldfish (Chang *et al.* 1990). Dosages used were previously tested for in vivo effects on GH and GTH-II serum levels (Chang et al. 1983a; Chang and Peter 1983 b&C; 1984; Wong et al 1993a&b).

Treatments

Injections

All injections were given at 9:00 AM and forebrains (see Figure 2-2) were collected at 2:00 PM, 5 hrs after initial injection. For time course trials, samples were collected at 2 hrs, 5 hrs and 8 hrs post injection. 16 fish were used for each time point, 8 injected with control vehicle and 8 injected with the drug being tested. Sex determination and separation before the experiment was not possible due to sexual immaturity of the fish.

Steroid Implantation

Solid silastic pellets containing Testosterone (T), Estradiol (E2), or no steroid (Blank) were a kind gift from Dr. Vance Trudeau (University of Ottawa) and manufactured as previously described (Pankhurst *et al.* 1986) with the elastomere containing 100 mg/g of steroid. Fish received a steroid dose of 100 ug/g body weight. Under tricaine methanesulphonate anaesthesia, pellets were implanted intraperitoneally (i.p) through a 2-3 mm incision in the body wall. Regressed fish were used to eliminate the confounding variable of endogenous sex steroids; 15 fish were used for each treatment. The treatments lasted 5 days post implantation and then the telencephalic-preoptic area and hypothalamus were removed for RNA extraction. Sex determination was not possible before implantation.

Seasonal Sampling

Samples were taken three times in the year, April 3, July 31, and Dec 21. Forebrain regions (see Figure 2-2) of 20 fish were collected, immediately frozen on dry ice, and stored at -70°C until total RNA was extracted. All total RNA extractions were done within the same one week period. All RNA samples were normalized to April males,

which was present for all hybridizations, to control for changes in hybridization conditions that may occur.

Quantitative Analysis of mRNA Levels by Northern Analysis

Northern analysis was performed in the same manner as for distribution studies. Membranes were then exposed to the phosphoimager and analyzed using ImageQuant program. The quantity of sample was controlled for by doing a ratio of PSS expression to α-tubulin expression. From quantitative analysis of α-tubulin expression in relation to total RNA concentration per sample and from previous work by Mahmoud *et al.* (1995) it is assumed that the experimental manipulations have no effect on α-tubulin expression. These ratios, for injection and implantation experiments, were then expressed as a percent of control animals. Expressing the values as % control allows for a comparison between membranes hybridized at different times, with a different probes. Statistical analyses was done using a student's *t*-test. Differences were considered significant when $P<0.05$.

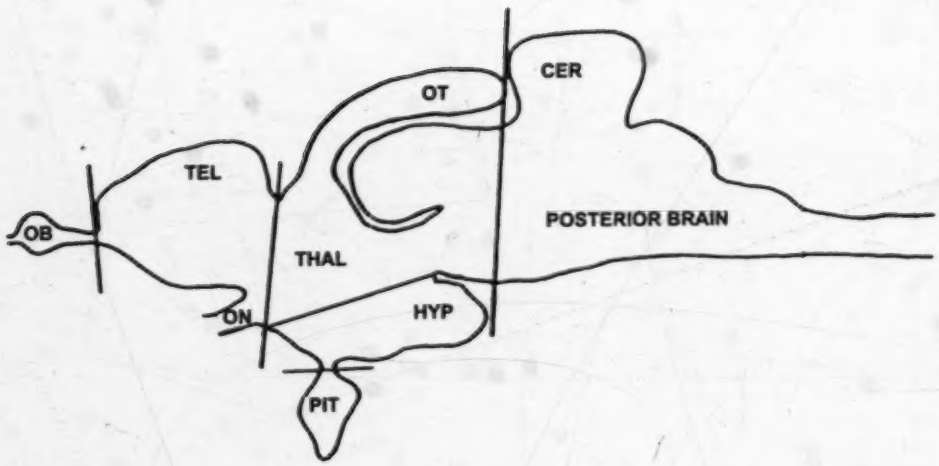

Figure 2-1: A schematic diagram of the goldfish brain divided into 6 distinct regions. **OB**-olfactory bulbs; **TEL**-telencephalon-preoptic region including the optic nerve (**ON**); **HYP**-hypothalamus; **OT-THAL**-optic tectum-thalamus; **Posterior brain**-cerebellum-medulla-spinal cord and **PIT**-pituitary.

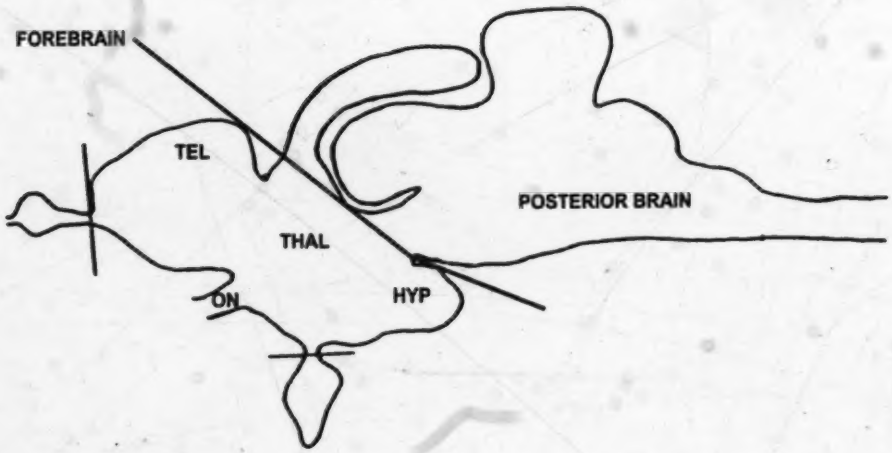

Figure 2-2: A schematic diagram of the goldfish brain defining the forebrain region. **TEL**-telencephalon-preoptic area, including the optic nerve (**ON**); **THAL**-thalamus; and **HYP**-hypothalamus.

CHAPTER 3

Results

Complementary DNA Encoding PSS-I^2

Goldfish Preprosomatostatin-I^2

Screening of a goldfish brain cDNA library with a probe for the coding region of PSS-II identified two positive colonies which hybridize to the probe. After secondary screening, two positive colonies were characterized by nucleotide sequence analysis. The identity of the sequence suggests a novel cDNA, PSS-I^2, since the deduced peptide sequence contains SS-14 at its C-terminus with a proline substitution at position 2 of SS-14. Both clones contained the identical nucleotide sequence for PSS-I^2.

The 701 nucleotide sequence of goldfish PSS-I^2 cDNA is shown in Figure 3-1, along with the predicted amino acid sequence of the SS precursor. The cDNA is comprised of 104 base 5'-untranslated region, 336 base pairs of open reading frame, and a 260 base 3'-untranslated region, which contains a modified polyadenylation signal (ATTAAA) and a poly (A) tail. The nucleotide sequence of the open reading frame predicts a goldfish PSS-I^2 of 111 AA. The deduced PSS-I^2 molecule consists of a putative signal sequence of 19 AA from position +1 to 19 (Figure 3-1). The signal sequence has features common to other signal peptides. The precursor also contains a number of putative enzyme cleavage recognition sites (Douglas *et al.* 1984). These include an arginine monobasic site at position +82 potentially yielding a 29 AA peptide, and at +87 potentially yielding a 26 AA peptide, as well as an Arginine-Lysine dibasic site at position +96 and +97, potentially yielding [Pro^2]SS-14 (Figure 3-1).

Phylogenetic Analysis of the Known PSS Coding Sequences

Sequence homology between the three known goldfish PSS cDNA coding regions were compared using Clustal W. PSS-I^1 and PSS-I^2 are 42% homologous, PSS-I^1 and PSS-II are 41% homologous and PSS-II and PSS-I^2 are 36% homologous. Figure 3-2 shows a phylogenetic tree based on the multiple alignment of the known PSS coding sequences. The phylogenetic relationships show a higher similarity between goldfish PSS-I^1 coding region and the cDNA coding regions for SS-14 peptide in other species. Similarly PSS-II is most closely related to cDNA encoding larger forms of SS in other species. PSS-I^2 is most closely associated with the coding region for the known frog variant of SS-14, [Pro^2,Met^{13}]SS-14. When the frog variant and PSS-I^2 coding regions are compared the sequence similarity is 50%.

Sequence for PSS-II Gene

To identify the goldfish SS gene structure and confirm the SS-28 cDNA sequence derived from 3' and 5'-RACE, PCR with the specific primer sets for PSS-I^1, PSS-I^2 and PSS-II was performed on liver genomic DNA (Figure 3-3). Results, after separation by size on an electrophoresis gel, showed that SS-14 and SS-28 have a genomic sequence length, minus the promotor region, of close to 1200 bp. [Pro^2]SS-14 has a genomic length close to 650 bp.

Further subcloning and sequencing of the SS-28 fragment, amplified with PSS-II F and PSS-II R specific primer set, confirmed the cDNA sequence found through 3', 5'-RACE (Figure 3-4). This partial genomic SS-28 sequence, 1245 bp total length, has one intron, 845 bp long, with a coding region of 371 bp and a single basic processing site, arginine, for the SS-28 peptide. A dibasic processing sight is also present for [Glu^1, Try^7, Gly^{10}]SS-14 mature protein. The promoter region of the gene has not been isolated.

Differential Expression of PSS Genes in Different Tissues and Brain Regions

Body

RT-PCR was performed on cDNA samples from six body organs: muscle, liver, kidney, brain, gonad, and gastrointestinal tract (GI) (Figure 3-5). PSS-I^1 was detected in the kidney, brain and GI tract. PSS-I^2 was detected in the liver and brain. PSS-II was detected in the liver brain and GI tract. β-actin was detected in all of the tissues examined verifing the quality and integrity of the cDNA samples.

Pituitary

Northern analysis of whole pituitary total RNA samples detected only a faint band for PSS-I^1 (Figure 3-6). Specificity of individual probes was observed for the three probes. RT-PCR was also performed on pituitary cell mRNA to confirm the location of PSS-I^1 in the pituitary and not nerve fibers originating in the brain. Results of ethidium bromide staining of the agarose gel and southern analysis confirmed the presence of PSS-I^1 in the pituitary cells with no signals for PSS-I^2 and PSS-II (Figure 3-7).

Brain

Southern analysis of RT-PCR products amplified from the brain of goldfish defines regions expressing SS genes (Fig 3-8). PSS-I^1 and PSS-I^2 were detected in the telencephalon-preoptic area, hypothalamus, thalamus-optic tectum area and posterior brain. PSS-I^2 is also found in the olfactory bulbs. PSS-I^2 was clearly visualized in all brain areas with ethidium bromide staining of the agarose gel. Southern blot analysis for PSS-I^2, however, gave too strong a hybridization signal in all areas to give a distinct band. PSS-II is localized in the telencephalon-preoptic region, hypothalamus, and thalamus. No

cross hybridization was seen for each of the three probes.

Northern blot analysis was generally consistent with RT-PCR and southern results (Fig 3-6). However, PSS-I^2 is not detected in the posterior brain by northern blot analysis suggesting the amount of mRNA is undetectable without amplification in that area. PSS-I^2, however, is more clearly defined in northern blot analysis confirming its presence in the olfactory bulbs, telencephalon-preoptic region, hypothalamus and thalamus. PSS-I^1 distribution remained unchanged with PSS-I^1 expression detected in all areas except the olfactory bulbs. All five brain total RNA samples were also hybridized with α-tubulin as a control for sample integrity.

Functional Characteristics of [Pro^2]SS-14

Inhibition of pituitary GH secretion

Two min pulses of increasing doses of [Pro^2]SS-14 resulted in a rapid and dose-dependent decrease in GH release from goldfish pituitary fragments (Figure 3-9). SS-14 and [Pro^2]SS-14 were tested simultaneously with the ED_{50} of the two curves at 2.33±2.17 nM and 3.56±2.43 nM respectively, showing no significant difference. GH secretion after each pulse returned to baseline levels, with increasing duration of the inhibition response with increasing concentrations. At 100 nM, the GH baseline secretion level recovered only after the full 60 minutes had passed.

Effect on sGnRH-stimulated GH release

In sexually recrudescent fish, three 5 min pulses of sGnRH given at 30 min, 90 min and 150 min following the start of the experiment, produced threee GH responses which decreased in size with successive sGnRH treatments (Figure 3-10 A&B). The GH

response decreases significantly from 239.75±25.8%, first pulse, to 190.08± 38.36%, second pulse, to 116.90±38.36%, third pulse. One hour continuous perifusion of 100 nM [Pro^2]SS-14, commencing 30 min before and ending at 30 min after the second pulse was tested in parallel. The GH release response to this second GnRH pulse was completely blocked and GH levels declined and GH levels were almost zero during the continuous [Pro^2]SS-14 perifusion. The magnitude of the response to the third pulse of 94.58±13.77%, 30 min after [Pro^2]SS-14 removal, was significantly lower than the initial response of 257.25% ±35.59 but not significantly lower than the response to the third pulse in the control columns. Exposure to [Pro^2]SS-14 had no prolonged effects on GnRH stimulated GH release.

Effect on D1 Agonist-Stimulated GH Release

Pituitary fragments, prepared from sexually recrudescent fish, were used to study the actions of [Pro^2]SS-14 on DA D_1 receptor agonist-stimulated GH release (Figure 3-11 A&B). Three control pulses of SKF 38393, applied with 1.5 hours between pulses, stimulated GH responses that showed no significant decrease in magnitude with repeated exposure to the D1 agonists. The mean GH response was 690.01± 58.29% . In the presence of 1 hour continuous 100 nM [Pro^2]SS-14 perifusion, basal GH release was significantly suppressed to 85.6% ± 3.59 below baseline of 100%. The second SKF 38393 pulse, administered during continuous [Pro^2]SS-14 treatment, caused no change in the already depressed baseline GH secretion. After removal of [Pro^2]SS-14, GH levels returned to near the initial baseline levels, with no significant difference in the average baseline levels compared to pretreatment levels at the onset. Previous exposure to [Pro^2]SS-14, however, inhibited the GH response to the third SKF 38393 pulse.

Regulation of Somatostatin Gene Expression

Seasonal Alteration in Somatostatin Gene Expression

PSS-I^1

Forebrain samples were used to measure the changes in mRNA expression in male and female goldfish in April (sexually mature), July (sexually regressed) and December (sexually recrudescent; see Table 3-1 for the Gonadal Somatic Index (GSI) at different times in the year). All values were normalized to α-tubulin expression and are presented as values relative to April males=100%. No sexual differences in mRNA levels were seen in July or December samples (Figure 3-12A). In April, however, female fish had significantly higher levels of PSS-I^1 mRNA expression than males sampled at any time, as well as compared to females in July. Females in December also exhibited significantly higher levels of PSS-I^1 mRNA than in July, but was not significantly different from values in April. Males showed no difference in expression between April, July and December. Therefore only females show seasonal variation in PSS-I^1 mRNA expression.

PSS-I^2

Forebrain samples showed significant differences between males and females in both April and December (Figure 3-12B). In December, males expressed significantly more PSS-I^2 than females, with no significant difference between July and December expression rates for males. Female expression rates were the lowest in December with increasing expression in April. April females express significantly higher levels of PSS-I^2 than do April males. July had the highest levels of PSS-I^2 expression for both males and females. The results are presented as values relative to April males=100%.

PSS-II

Measurements of PSS-II mRNA expression from the preoptic-hypothalamic area show significant differences between males and females at sexual maturity in April (Figure 3-12C). PSS-II mRNA levels in females is significantly higher than PSS-II mRNA levels in males at sexual maturity. Seasonal expression levels are the highest in July for both females and males. December expression levels are not significantly different from levels in April males for both sexes.

TABLE 3-1: Gonadal somatic Index (GSI) of goldfish collected in April, July, and December.

	Female	**Male**
April 3	6.5%±0.68	3.5%±0.31
July 31	0.11%±0.069	0.21%±0.059
December 21	4.06%±0.91	2.6%±0.33

Regulation of Somatostatin Gene Expression by GnRH

sGnRH, 0.01 µg/g body weight, was injected interperitoneally (i.p.) in sexually regressed goldfish and forebrain samples were taken 5 hrs post injection (Figure 3-13). Using northern blot analysis only PSS-II mRNA levels show a significant increase at five hours post injection ($P<0.05$). PSS-I^1 and PSS-I^2 showed no changes compared to levels in controls.

Dopaminergic Regulation of Somatostatin Gene Expression

Effect of catecholamines on SS gene expression

α-MPT, a blocker of catecholamine synthesis, was injected i.p. in sexually regressed goldfish at 300 mg/g body weight and the forebrain extracted 5 hrs post-injection. Northern blot analysis of PSS mRNA in the forebrain shows a significant decrease in PSS-I^2 and PSS-II mRNA compared to controls (Figure 3-14). No significant changes were seen in PSS-I^1 mRNA expression in the forebrain.

Effect of Dopamine Agonists and Antagonists on PSS mRNA Expression in the Forebrain

1. Apomorphine

Apomorphine, 20 µg/g body weight, was injected i.p. in sexually regressed goldfish and forebrain samples were taken 5 hrs post injection. Northern analysis of PSS mRNA in the forebrain shows a significant decrease in PSS-I^1, PSS-I^2 and PSS-II mRNA expression compared to controls (Figure 3-15).

2. D_1 receptors

IP injection of 40 µg/g SKF 38393, a D1 agonist, was given to sexually regressed fish, and forebrain samples taken at 5 hrs post injection. Analysis by northern blot shows that PSS mRNA expression was decreased with respect to control fish injected with propylene glycol. The magnitude of the effect varied between the different PSS mRNA. Injection of SKF 38393 caused a significant decrease in all three forms of PSS mRNA (Figure 3-16).

To determine the effects of SKF 38393, on PSS mRNA expression over time, samples were collected at 2, 5 and 8 hrs after injection (Figure 3-17). PSS-I^1 mRNA decreased at 5 hrs post injection and no changes from control levels were seen at 2 hrs and 8 hrs. PSS-I^2 showed no effects in mRNA expression at 2 hrs, with a significant increase at 5 hrs post injection ($P<0.05$). At 8 hrs, however, PSS-I^2 mRNA levels fell well below control mRNA expression levels. PSS-II showed an initial increase in mRNA expression at 2 hrs followed by the previously defined decrease in mRNA levels at 5 hrs. Sampling at 8 hrs post injection saw PSS-II levels return to control value levels.

The effect of i.p. injection with the D_1 antagonist, SCH 23390, at 40 µg/g body weight were tested to determine if there was a tonic inhibitory or stimulatory action of DA on PSS mRNA expression (Figure 3-18). PSS-I^1 and PSS-II mRNA were significantly increased at 5 hours post IP injection of SCH 23390. However, PSS-I^2 mRNA

expression was significantly decreased at 5 hrs post injection.

3. D_2 receptors

The D_2 agonist, LY 171,555, was injected i.p. in sexually recrudescent goldfish and forebrain samples were analyzed 5 hrs post injection. PSS-I^1 and PSS-II mRNA expression decreased significantly, whereas PSS-I^2 again responded in a unique fashion showing an elevation in mRNA expression 5 hrs post injection (Figure 3-18 and 3-19).

The effects of i.p. injection of LY 171 555 were also tested in more sexually mature female and male fish (Figure 3-20). No significant differences in PSS-I^1 mRNA expression were seen between D_2 agonist treated fish and controls in both male and female fish. This is different from recrudescent fish which showed a significant decrease in PSS-I^1 mRNA expression. PSS-I^2 mRNA expression remains elevated above control as in recrudescent fish. PSS-II shows an increase in expression with LY 171,555 i.p. injection in mature male and female fish; this was completely opposite to the decrease in expression seen in recrudescent fish.

The time-dependent effects of i.p. injection of LY 171,555 on PSS mRNA expression were measured at 2 hrs, 5 hrs and 8 hrs post injection. The fish used were in late gonadal recrudescence and therefore female and male fish were analyzed separately. PSS-I^1 mRNA expression was increased above control levels at 2 hrs and then decreased back to control levels at 5 and 8 hour time points in both males and females (Figure 3-21 and 3-22). No effect on PSS-I^1 mRNA levels were seen, similar to previously described experiments on more mature fish sampled at 5 hrs post injection.

In females, injection of LY 171,555 did not cause significant changes in PSS-I^2 mRNA expression at 2 hours and levels increased above controls at 5 and 8 hrs post injection (Figure 3-21). Males showed a significant decrease at 2 hrs with an increase above controls at 5 and 8 hrs post injection (Figure 3-22).

PSS-II mRNA expression in female fish was decreased significantly at 2 hrs following injection of LY 171,555, with an increase above control levels at 5 hrs and a

return to control levels at 8 hrs (Figure 3-21). Male fish, however, showed no significant changes from control levels at 2 hrs (Figure 3-22). An increase in PSS-II mRNA expression above control levels was found at 5 hrs, as previously described, and maintained high levels at 8 hrs post injection.

Recrudescent fish given i.p. injection of the D_2 antagonist, pimozide, show opposite effects to the agonist in affecting PSS-I^1 and PSS-II mRNA levels, with an increase in mRNA expression at 5 hrs post injection (Figure 3-18). There were no changes in expression of PSS-I^2 mRNA under the same conditions.

Regulation of Somatostatin Gene Expression by Gonadal Steroids

Sexually regressed female fish were used for this study (GSI=1.27±0.26). Separation of male and female fish was not possible before implantation of the pellets and although 15 fish were implanted with each variety of pellet. There was also degredation in some samples due to storage at –20 C therefore the only usable samples were 6 female samples. PSS-II was not tested for in the preoptic area because it could not be detected through northern blot in this area. PSS-I^2 was not tested for in the hypothalamus due to the insufficiant quantity of sample in the hypothalamus to test all three probes.

Sex steroid implantation of T for five days showed no significant effect on PSS-I^1 mRNA expression in the telecephalic-preoptic region (Figure 3-23). A significant increase in PSS-I^1 mRNA expression was seem in E_2 treated goldfish. Both estradiol and testosterone implanted goldfish showed a significant increase in PSS-I^1 mRNA values in the hypothalamus (Figure 3-24). There was a significant increase in PSS-I^2 mRNA expression in the telencephalic-preoptic region after E_2 and T treatment (Figure 3-23). PSS-II mRNA expression also increased with both E_2 and T as compared to fish implanted with a blank pellet ($P<0.05$; Figure 3-24). All values were normalized to α-tubulin expression and expressed as % control.

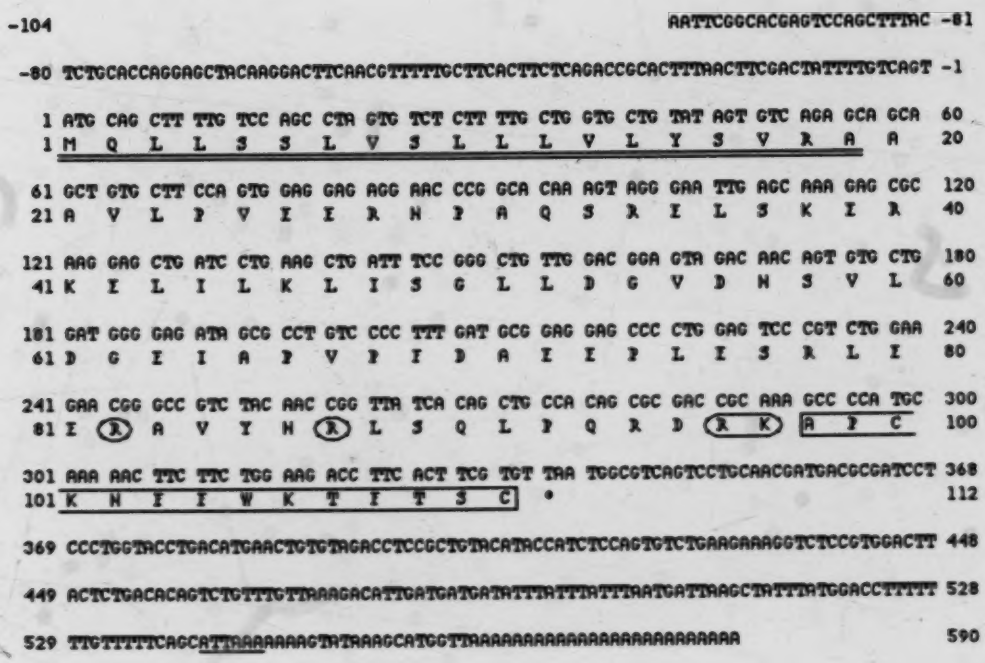
-104
AATTCGGCACGAGTCCAGCTTTAC -81
-80 TCTGCACCAGGAGCTACAAGGACTTCAACGTTTTGCTTCACTTCTCAGACCGCACTTTAACTTCGACTATTTGTCAGT -1
1 ATG CAG CTT TTG TCC AGC CTA GTG TCT CTT TTG CTG GTG CTG TAT AGT GTC AGA GCA GCA 60
1 M Q L L S S L V S L L L V L Y S V R A A 20
61 GCT GTG CTT CCA GTG GAG GAG AGG AAC CCG GCA CAA AGT AGG GAA TTG AGC AAA GAG CGC 120
21 A V L P V E E R N P A Q S R E L S K E R 40
121 AAG GAG CTG ATC CTG AAG CTG ATT TCC GGG CTG TTG GAC GGA GTA GAC AAC AGT GTG CTG 180
41 K E L I L K L I S G L L D G V D N S V L 60
181 GAT GGG GAG ATA GCG CCT GTC CCC TTT GAT GCG GAG GAG CCC CTG GAG TCC CGT CTG GAA 240
61 D G E I A P V P F D A E E P L E S R L E 80
241 GAA CGG GCC GTC TAC AAC CGG TTA TCA CAG CTG CCA CAG CGC GAC CGC AAA GCC CCA TGC 300
81 E R A V Y N R L S Q L P Q R D R K A P C 100
301 AAA AAC TTC TTC TGG AAG ACC TTC ACT TCG TGT TAA TGGCGTCAGTCCTGCAACGATGACGCGATCCT 368
101 K N F F W K T F T S C • 112
369 CCCTGGTACCTGACATGAACTGTGTAGACCTCCGCTGTACATACCATCTCCAGTGTCTGAAGAAAGGTCTCCGTGGACTT 448
449 ACTCTGACACAGTCTGTTTGTTAAAGACATTGATGATGATATTTATTTATTTAATGATTAAGCTATTTATGGACCTTTTT 528
529 TTGTTTTTCAGCATTAAAAAAAGTATAAGCATGGTTAAAAAAAAAAAAAAAAAAAAAAAA 590

Figure 3-1: cDNA sequence for PSS-I[2] isolated from the brain of goldfish through library screening. Double underline is the proposed signal peptide sequence, circled are mono and dibasic cleavage sites for possible mature peptide synthesis, Boxed is the proposed mature peptide produced (SS-14), and single underline is the polyadenilation signal sequence.

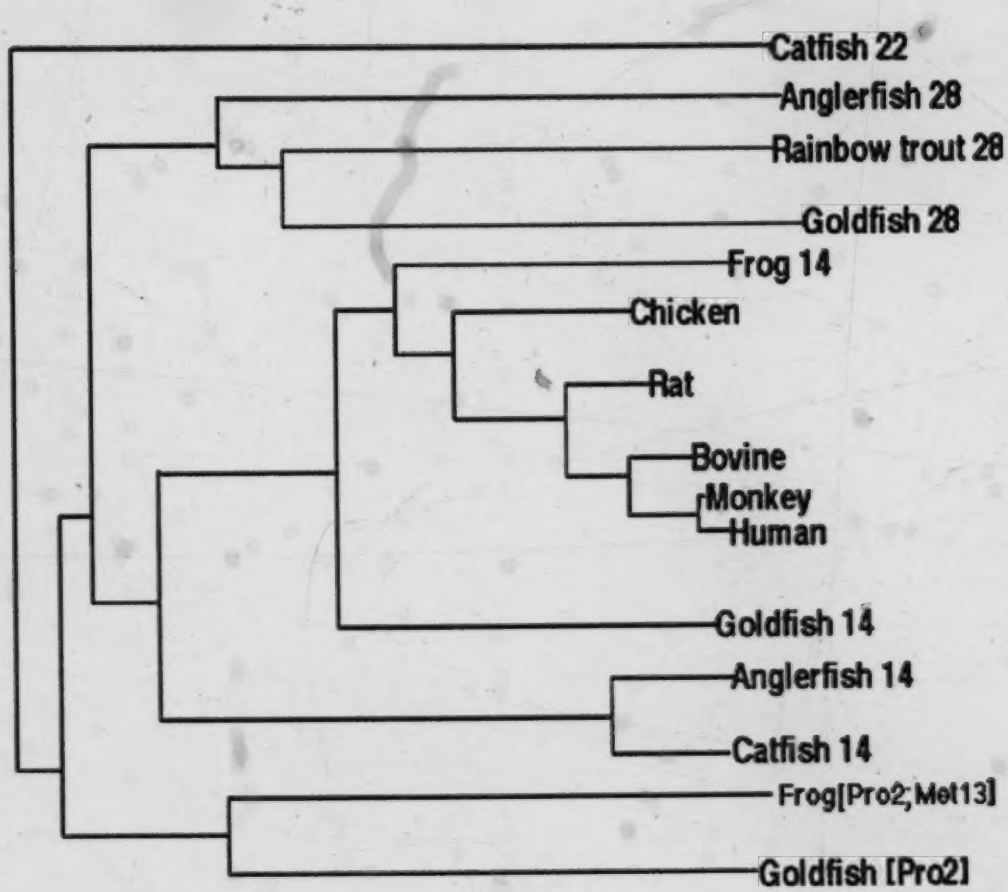

Figure 3-2: A phylogenetic tree describing the relationships between coding regions of all known SRIF mRNA regions from the NCI genebank data base. Using the computer program Clustal W, multiple sequence alignment, was carried out to compare the coding. See references in text.

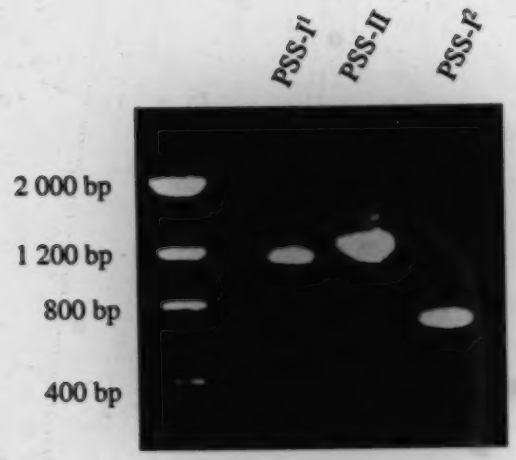

Figure 3-3: PCR products of genomic DNA amplified with specific primers for PSS-I[1], PSSI[2] and PSS-II, separated on a 1% agoarose gel.

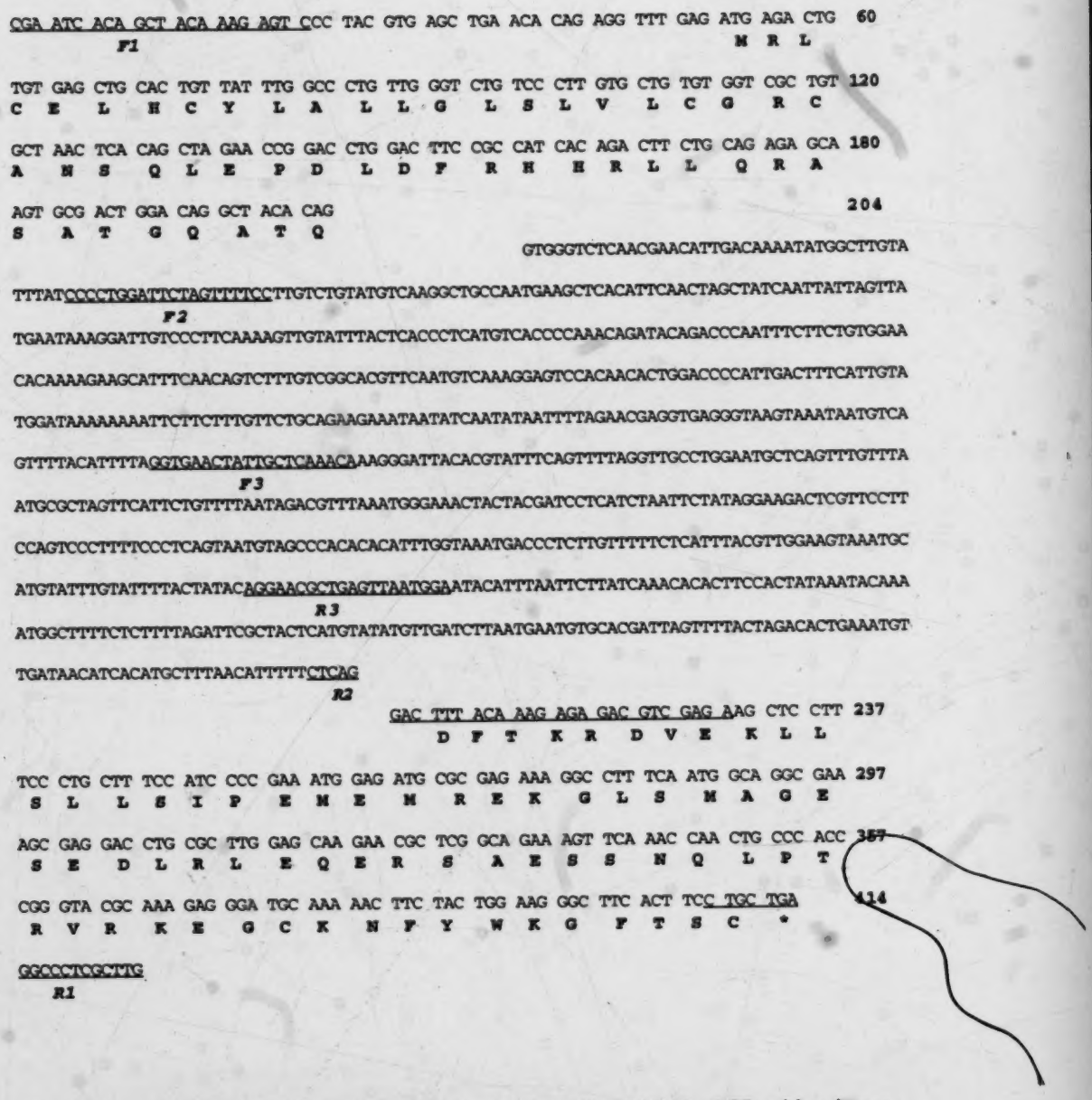
CGA ATC ACA GCT ACA AAG AGT CCC TAC GTG AGC TGA ACA CAG AGG TTT GAG ATG AGA CTG 60
F1
M R L
TGT GAG CTG CAC TGT TAT TTG GCC CTG TTG GGT CTG TCC CTT GTG CTG TGT GGT CGC TGT 120
C E L H C Y L A L L G L S L V L C G R C
GCT AAC TCA CAG CTA GAA CCG GAC CTG GAC TTC CGC CAT CAC AGA CTT CTG CAG AGA GCA 180
A N S Q L E P D L D F R H H R L L Q R A
AGT GCG ACT GGA CAG GCT ACA CAG 204
S A T G Q A T Q
GTGGGTCTCAACGAACATTGACAAAATATGGCTTGTA
TTTATCCCCTGGATTCTAGTTTTCCTTGTCTGTATGTCAAGGCTGCCAATGAAGCTCACATTCAACTAGCTATCAATTATTAGTTA
F2
TGAATAAAGGATTGTCCCTTCAAAAGTTGTATTTACTCACCCTCATGTCACCCCAAACAGATACAGACCCAATTTCTTCTGTGGAA
CACAAAAGAAGCATTTCAACAGTCTTTGTCGGCACGTTCAATGTCAAAGGAGTCCACAACACTGGACCCCATTGACTTTCATTGTA
TGGATAAAAAAAATTCTTCTTTGTTCTGCAGAAGAAATAATATCAATATAATTTTAGAACGAGGTGAGGGTAAGTAAATAATGTCA
GTTTTACATTTTAGGTGAACTATTGCTCAAACAAAGGGATTACACGTATTTCAGTTTTAGGTTGCCTGGAATGCTCAGTTTGTTTA
F3
ATGCGCTAGTTCATTCTGTTTTAATAGACGTTTAAATGGGAAACTACTACGATCCTCATCTAATTCTATAGGAAGACTCGTTCCTT
CCAGTCCCTTTTCCCTCAGTAATGTAGCCCACACACATTTGGTAAATGACCCTCTTGTTTTTCTCATTTACGTTGGAAGTAAATGC
ATGTATTTGTATTTTACTATACAGGAACGCTGAGTTAATGGAATACATTTAATTCTTATCAAACACACTTCCACTATAAATACAAA
R3
ATGGCTTTTCTCTTTTAGATTCGCTACTCATGTATATGTTGATCTTAATGAATGTGCACGATTAGTTTTACTAGACACTGAAATGT
TGATAACATCACATGCTTTAACATTTTTCTCAG
R2
GAC TTT ACA AAG AGA GAC GTC GAG AAG CTC CTT 237
D F T K R D V E K L L
TCC CTG CTT TCC ATC CCC GAA ATG GAG ATG CGC GAG AAA GGC CTT TCA ATG GCA GGC GAA 297
S L L S I P E M E M R E K G L S M A G E
AGC GAG GAC CTG CGC TTG GAG CAA GAA CGC TCG GCA GAA AGT TCA AAC CAA CTG CCC ACC 357
S E D L R L E Q E R S A E S S N Q L P T
CGG GTA CGC AAA GAG GGA TGC AAA AAC TTC TAC TGG AAG GGC TTC ACT TCC TGC TGA 414
R V R K E G C K N F Y W K G F T S C *
GGCCCTCGCTTG
R1

Figure 3-4: The partial goldfish genomic sequence encoding PSS-II. PCR with primers F1;R1 and genomic DNA from the liver amplified this 1245 bp DNA sequence. It includes one intron between base pair 204 and 205 of the coding region. Primers used for sequencing are underlined.

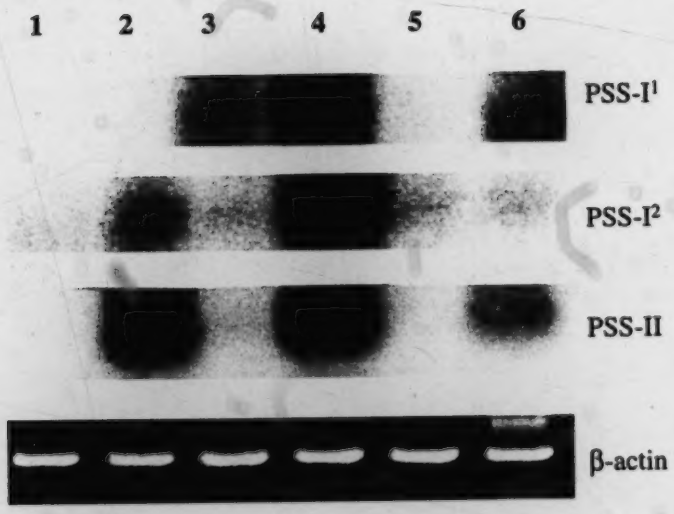

Figure 3-5: RT-PCR and Southern analysis of 6 regions of the body with probes and primers specific for PSS-I[1], PSS-I[2] and PSS-II. The six body regions include: (**1**) muscle, (**2**) liver, (**3**) kidney (**4**) brain (**5**) gonad and (**6**) gastro-intestinal tract. PCR using specific primers for β-actin was done for a control ensuring the presence of cDNA in all samples after first strand synthesis.

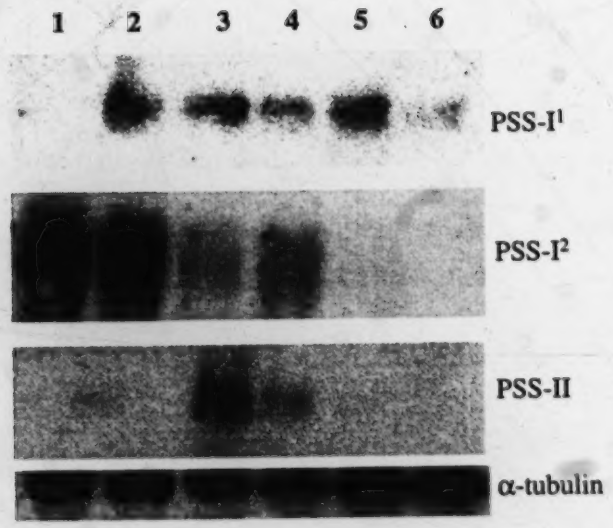

Figure 3-6: Northern analysis of five region of the brain and (6) the pituitary. The five brain areas include the (1) olfactory bulbs, (2) telencephalic-preoptic area, (3) hypothalamus, (4) thalamus-optic tectum area and (5) posterior brain. Probes specific for PSS-I[1], PSS-I[2] and PSS-II were used. αtubulin probe was used to control for the presence of total RNA in each sample.

Figure 3-7: RT-PCR products amplified from pituitary cell mRNA with specific primers for PSS-I^1, PSS-I^2 and PSS-II. Cholycystokinin (CCK) was amplified with specific primers to ensure cDNA integrity after first stand synthesis. All samples were run on a 1% agarose gel in the presence of ethidium bromide and photographed.

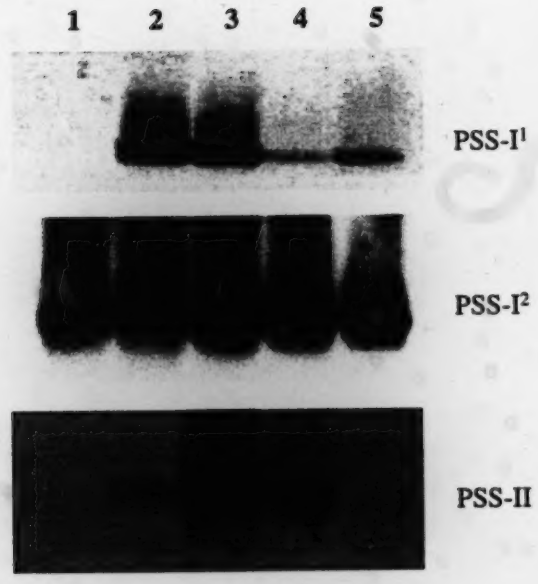

Figure 3-8: Southern analysis of five regions in the brain; (1) olfactory bulbs, (2) telencephalic-preoptic area, (3) hypothalamus, (4) thalamus-optic tectum, and (5) posterior brain. Probes specific for PSS-I^1, PSS-I^2 and PSS-II were used.

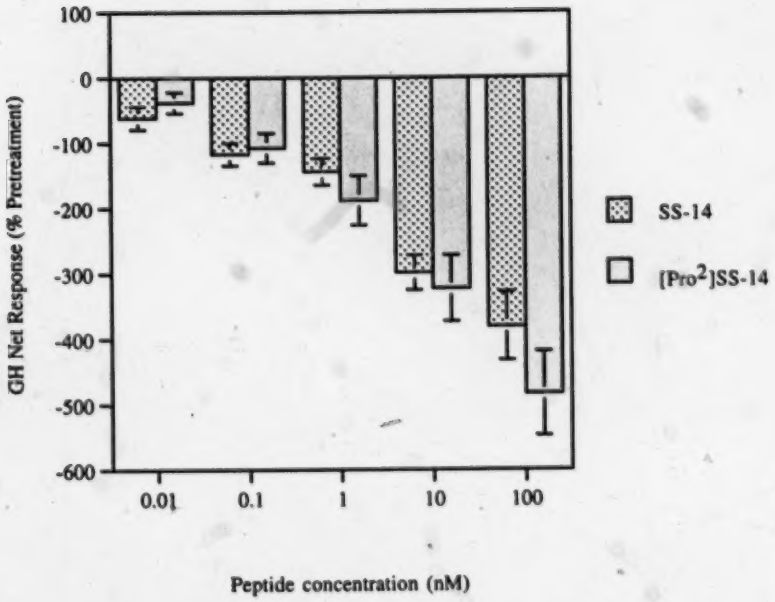

Figure 3-9: The effects of SS-14 and [Pro^2]SS-14 on pituitary GH secretion. GH net responses, quantitated as area under the curve, to 2 min pulses every hour, of 0.01-100 nM SS-14 or [Pro^2]SS-14 using perifused pituitary fragments from sexually recrudescent males and females. All data are expressed as mean +/-SEM (n=4-6).

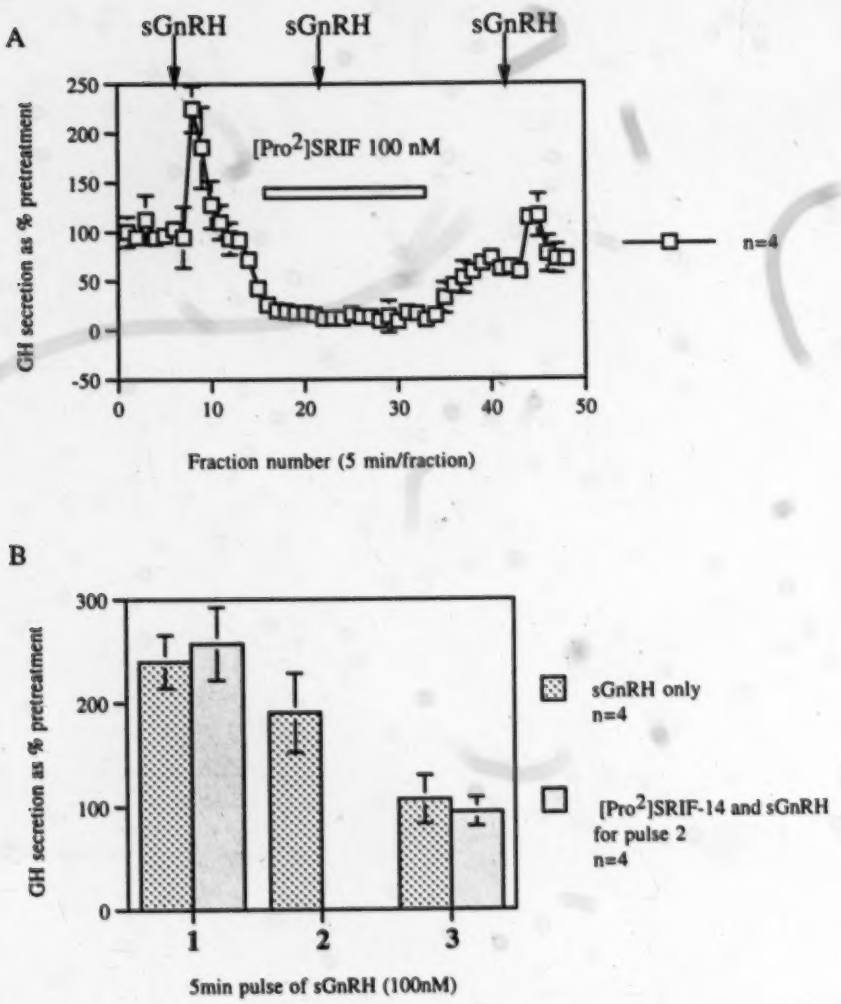

Figure 3-10: Effects of [Pro^2]SS-14 on sGnRH-stimulated GH release from perifused pituitary fragments of goldfish. sGnRH at 100nM dose was administered as 5 min pulses before, during, and after the 1-hr continuous perifusion of 100nM [Pro^2]SS-14. (A) GH release profile challenged with sGnRH before during and after 1 hr continuous [Pro^2]SS-14 perifusion. (B) The magnitude of GH secretion due to sGnRH, 100nM, challenge before, during and after 1hr continuous [Pro^2]SS-14 treatment.

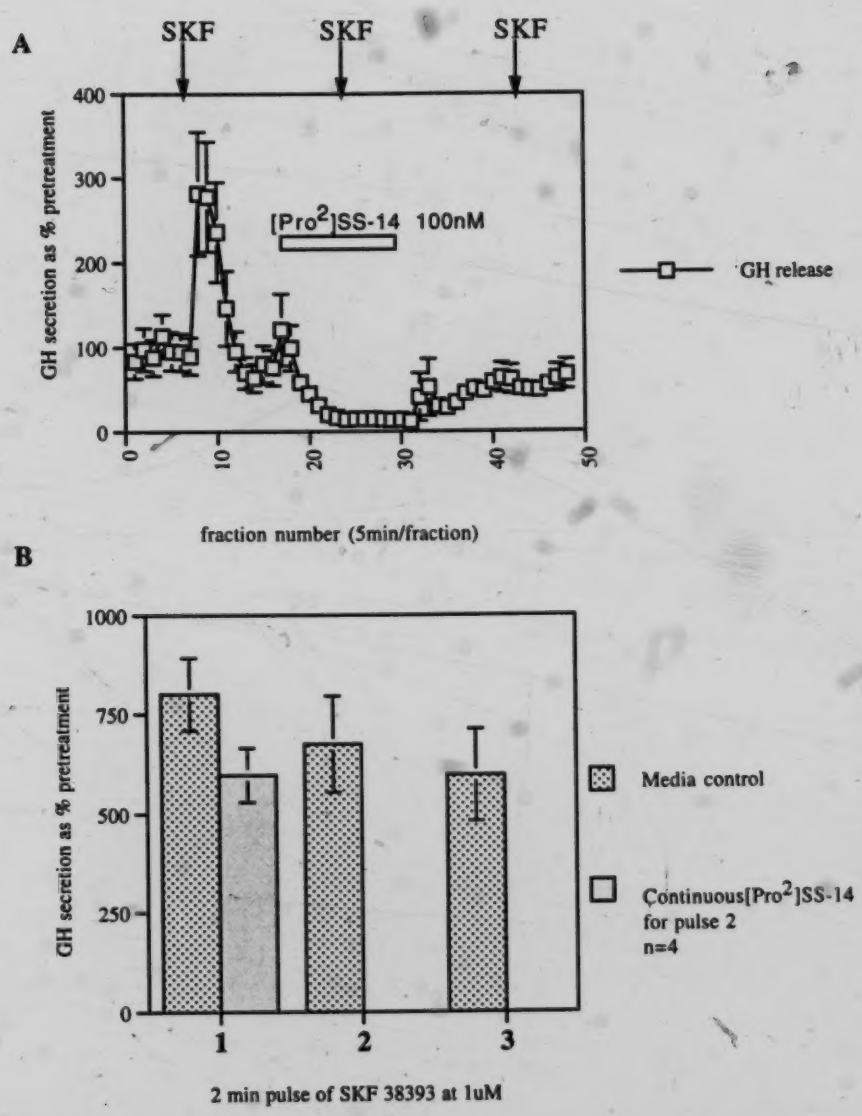

Figure 3-11: The effects of [Pro^2]SS-14 on dopamine-stimulated GH release from perifused pituitary fragments of goldfish. SKF 38393, a D1-agonist, was administered as 2 min pulses at 1uM before, during, and after 1hr continuous perifusion of 100nM [Pro^2]SS-14. (A) GH release profile exposed to pulses of SKF 38393 applied before, during and after 1hr continuous [Pro^2]SS-14 treatment. (B) The magnitude of GH secretion increase due to SKF 38393 before, during and after 1hr continuous [Pro^2]SS-14 perifusion.

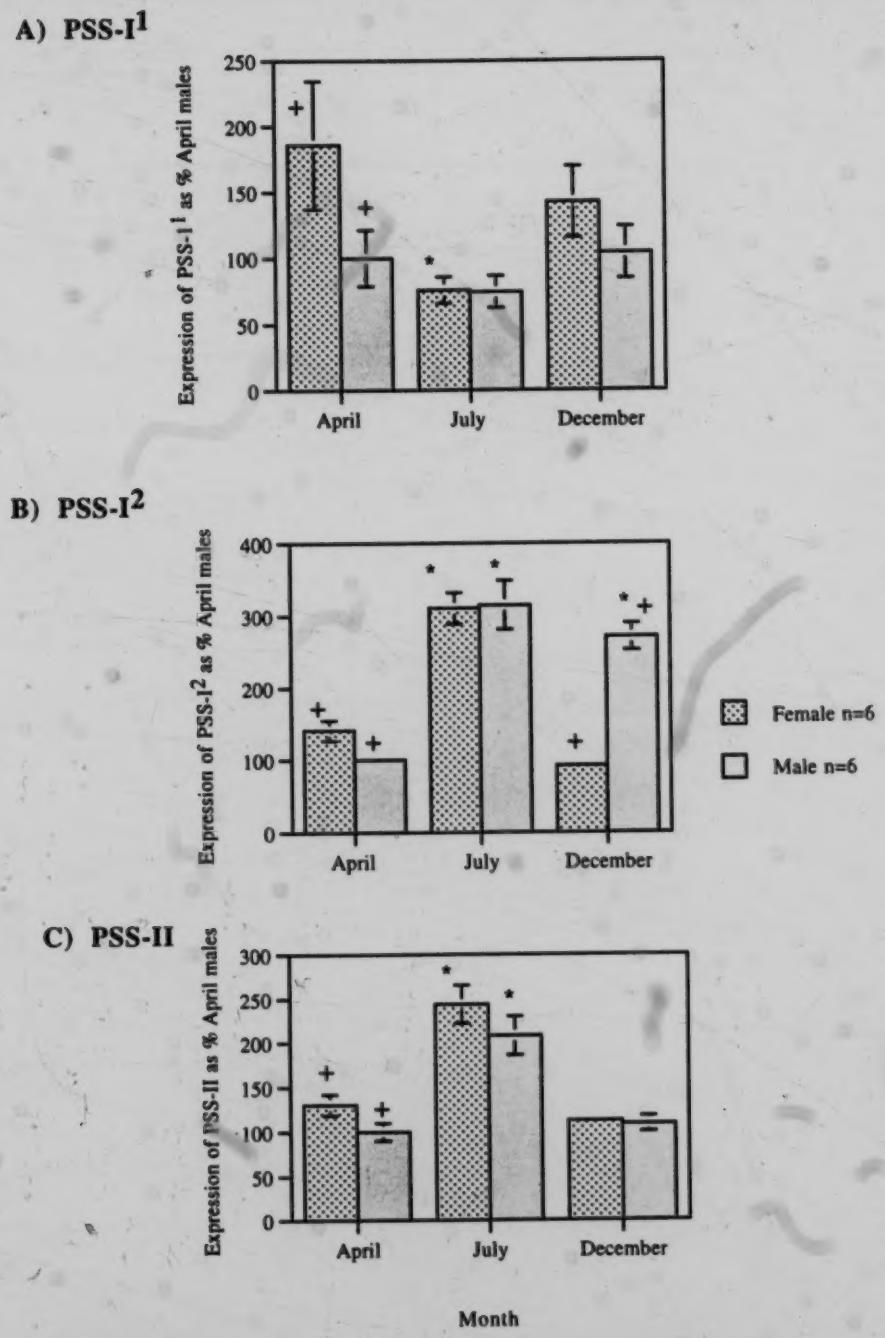

Figure 3-12: Seasonal (gonadal state) effects on the expression of A) PSS-I[1] B) PSS-I[2] and C) PSS-II mRNA in the forebrain of goldfish (see Table 1). (*) represents a significant difference from April values in the same sex. (+) represents a significant difference between sexes in the same month ($P < 0.05$).

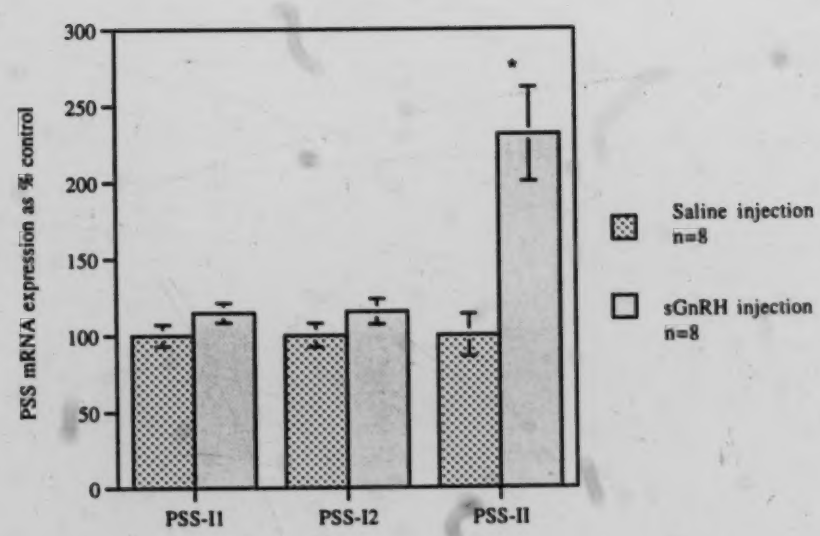

Figure 3-13: The effects of 0.01 ug/g body weight sGnRH by i.p. injection on PSS mRNA expression in the forebrain of sexually regressed male and female goldfish (GSI<1%); five hrs post injection. (*) shows a significant increase in PSS-II mRNA expression above control ($P<0.05$).

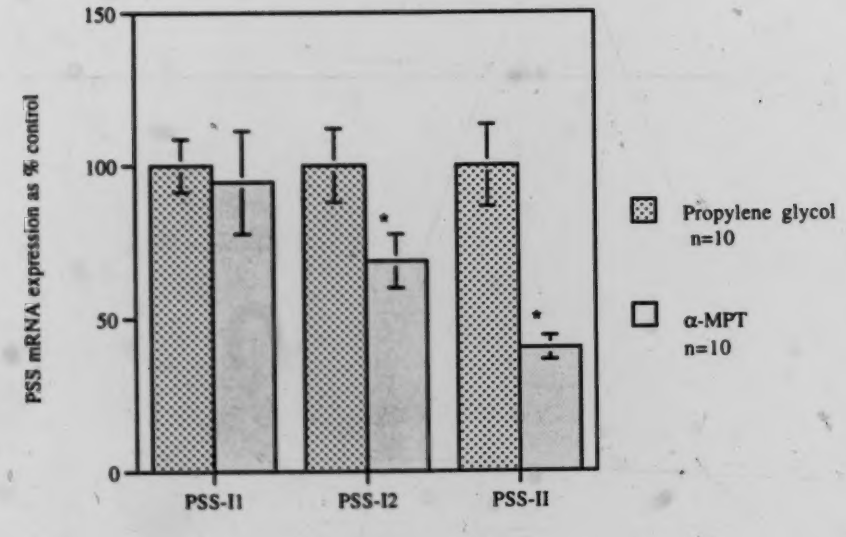

Figure 3-14: The effect of α-MPT i.p. injection, 300 ug/g body weight, on PSS mRNA expression in the foreberain of sexually regressed male and female goldfish (GSI<1%). (*) shows a significant difference from control for PSS-I^2 and PSS-II mRNA ($P<0.05$).

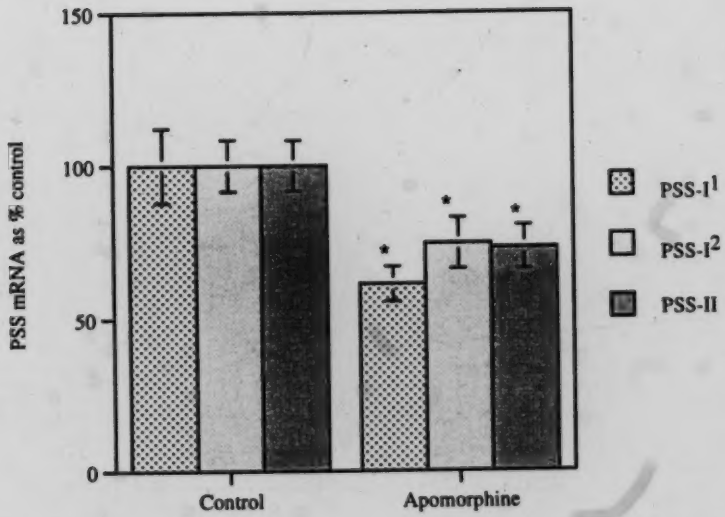

Figure 3-15: The effects of dopamine agonist, apomorphine, on PSS mRNA expression in the forebrain of sexually regressed male and female goldfish (GSI=1.2%+/-0.15 SEM). Apomorphine, 20 ug/g body weight, was administered by i.p injection and samples collected 5 hrs post injection. (*) shows a significant difference from control ($P<0.05$; n=8).

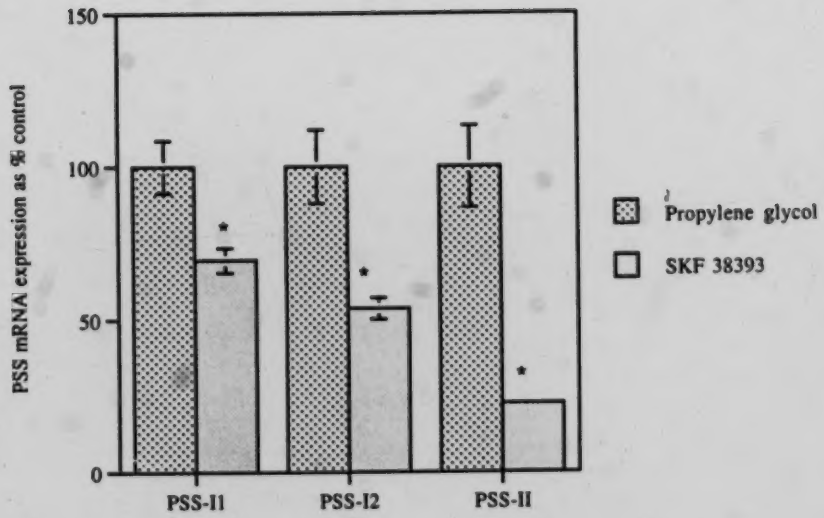

Figure 3-16: The effects of SKF 38393, a D_1 agonist, i.p. injection on PSS mRNA expression in the forebrain of sexually regressed male and female goldfish (GSI<1%) at 5 hours post injection. (*) Show a significant difference from control ($P<0.05$; n=10).

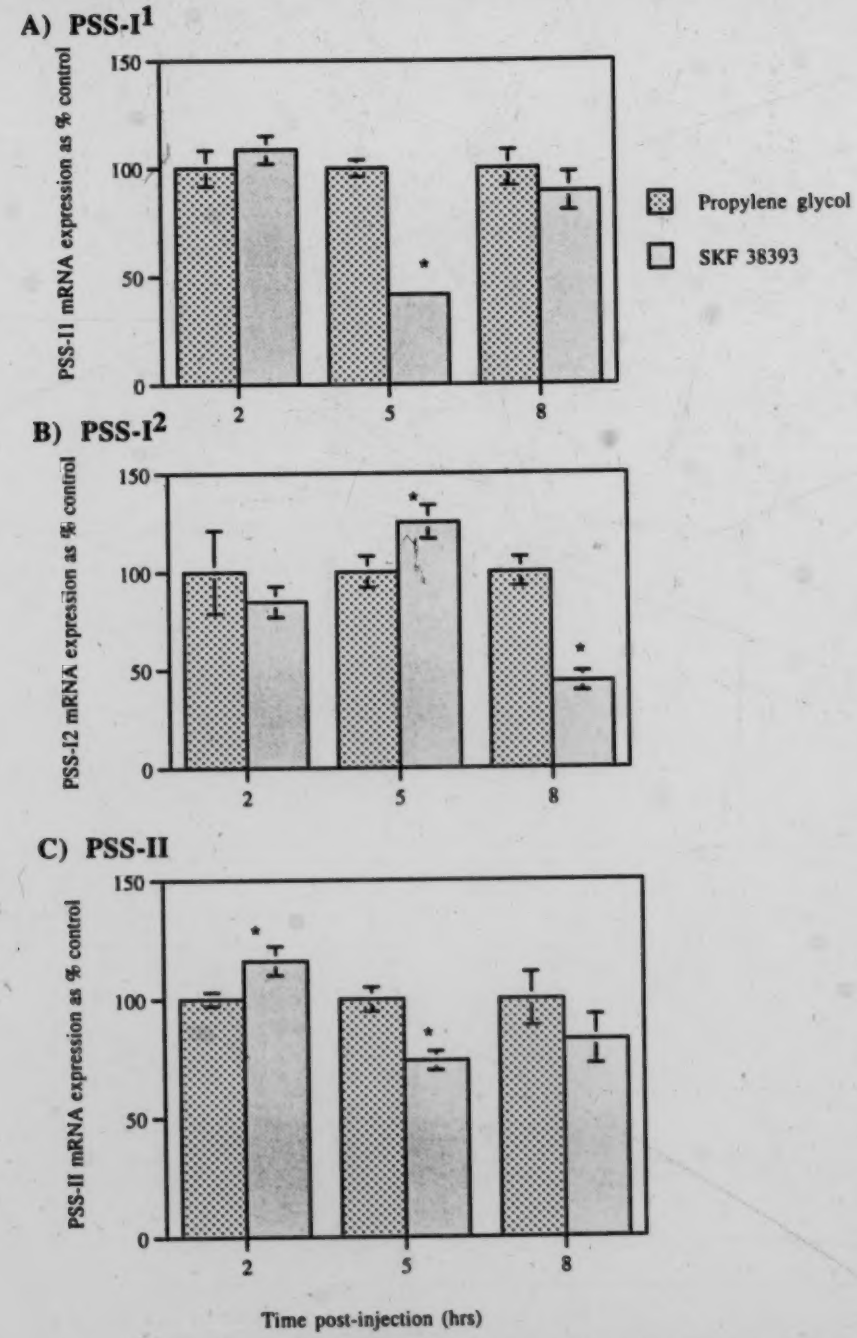

Figure 3-17: Time-dependent effects of SKF 38393, a D_1 agonist, on A) PSS-I^1, B) PSS-I^2 and C) PSS-II mRNA expression in the forebrain of sexually recrudescent male and female goldfish; GSI=2.1%+/-0.2 SEM. (*) shows a significant difference from control ($P<0.05$; n=8).

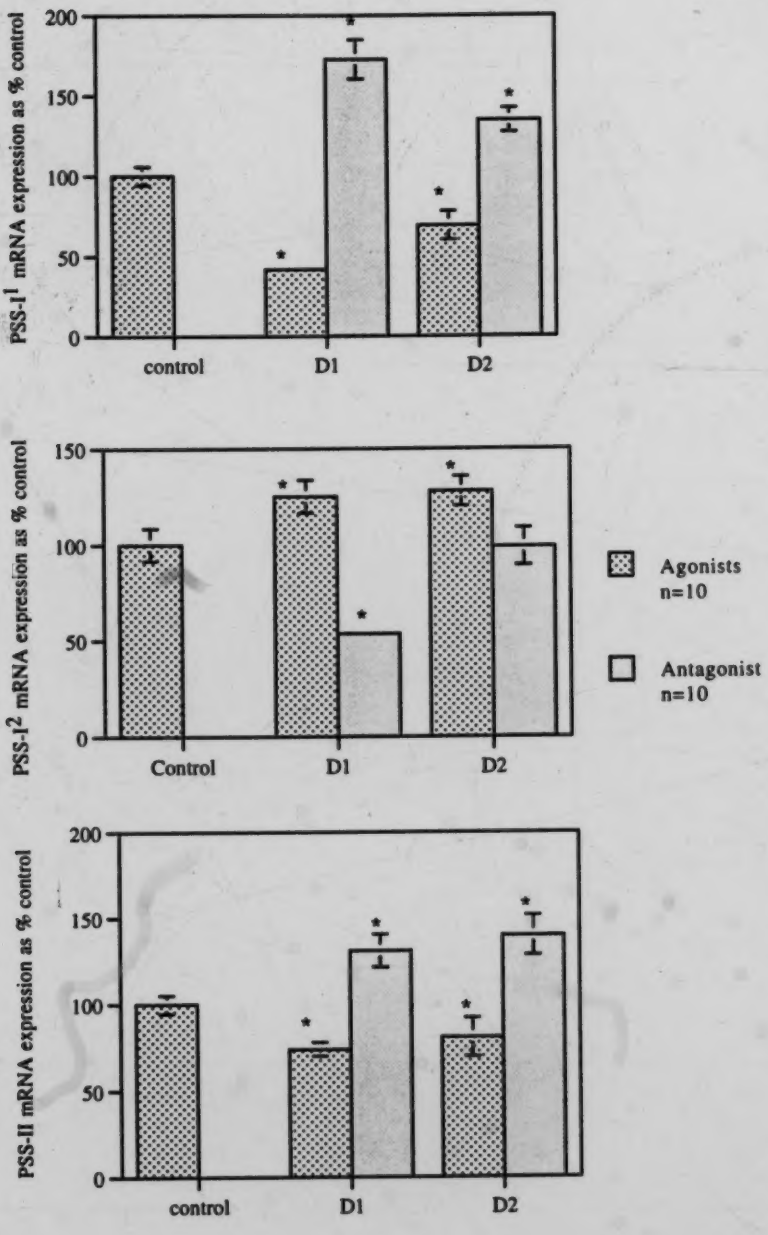

Figure 3-18 : The effects of i.p. injection of the D_1 agonist, SKF 38393, and antagonist, SCH 23390, and the D agonist, LY 171 555, and antagonist, Pimozide, on PSS mRNA expressio in the forebrain of goldfish at 5 hrs post injection. Sexually recrudescent male and female goldfish; GSI= 1.80%+/- 0.2 SEM. (*) shows a significant difference from control (propylene glycol injection; $P<0.05$)

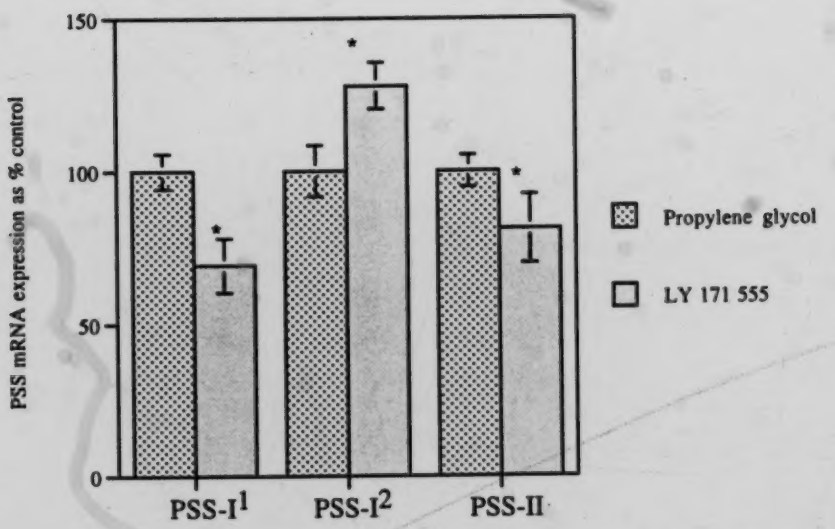

Figure 3-19: The effects of LY 171 555, a D_2 agonist on PSS mRNA expression in the forebrain of sexually recrudescent male and female goldfish (GSI=1.86%+/-0.15 SEM). (*) shows a significant difference from control ($P<0.05$; n=8).

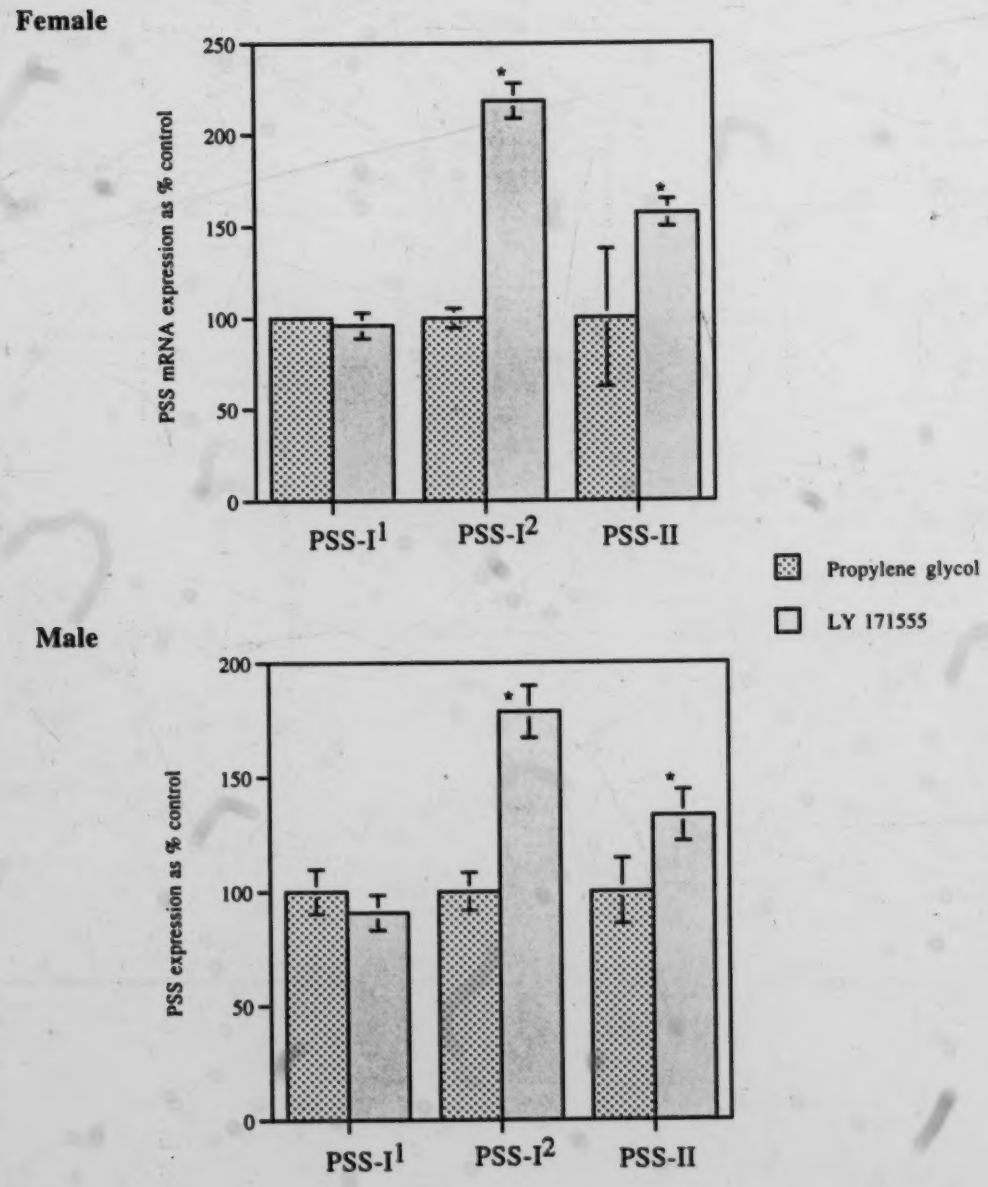

Figure 3-20: The effects of LY 171 555, a D2 agonist, i.p. injection on PSS mRNA expression, at 5hrs post-injection, in the forebrain of sexually mature female (GSI=7.05%+/-1.56 SEM) and male (GSI=3.98%+/-0.55 SEM) goldfish. (*) shows a significant difference from control ($P<0.05$; n=4).

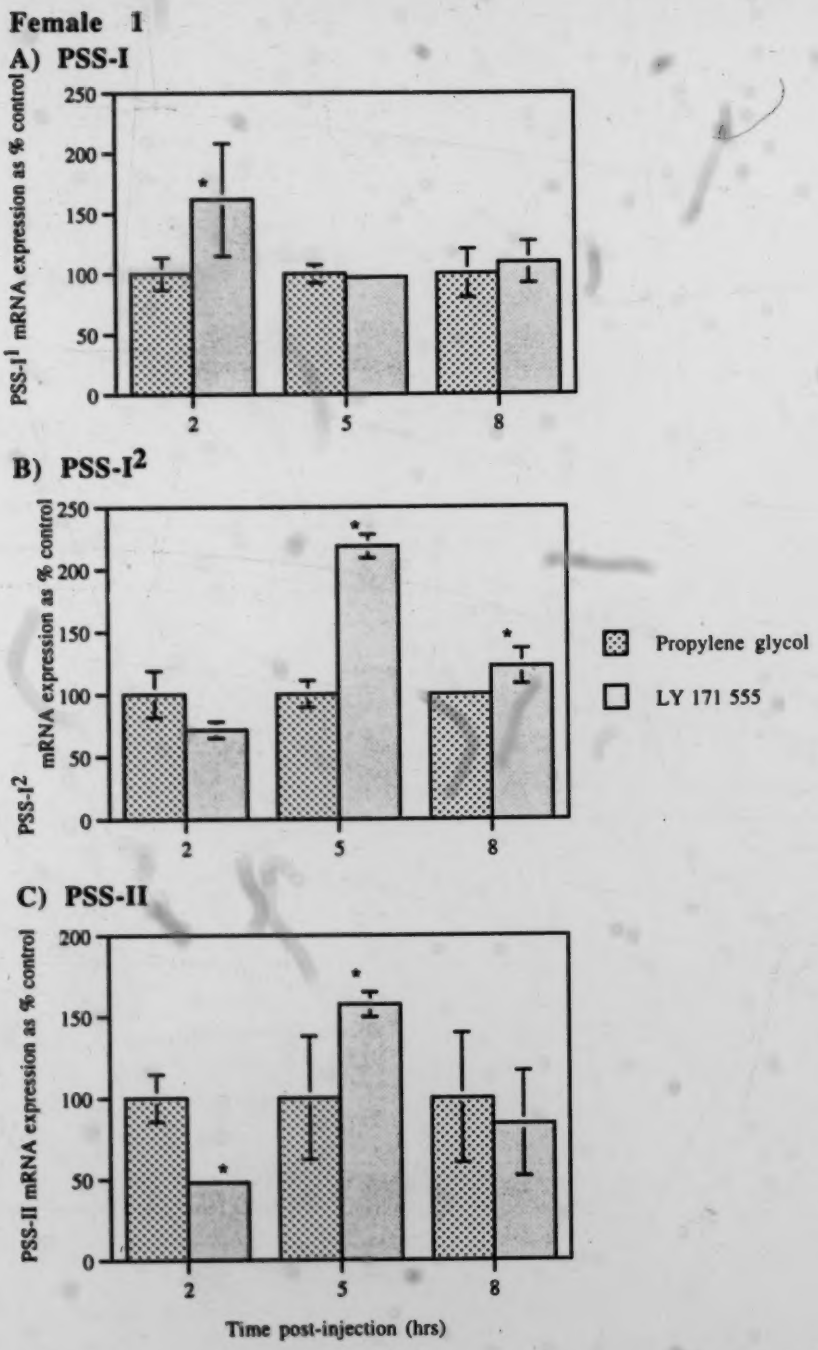

Figure 3-21: The effects of LY 171 555, a D agonist, on A) PSS-I[1] B) PSS-I[2] and C) PSS-II mRNA expression in the forebrain of sexually recrudescent female goldfish; GSI=5.33%+/-1.35SEM. (*) shows a significant difference from control ($P<0.05$; n=4).

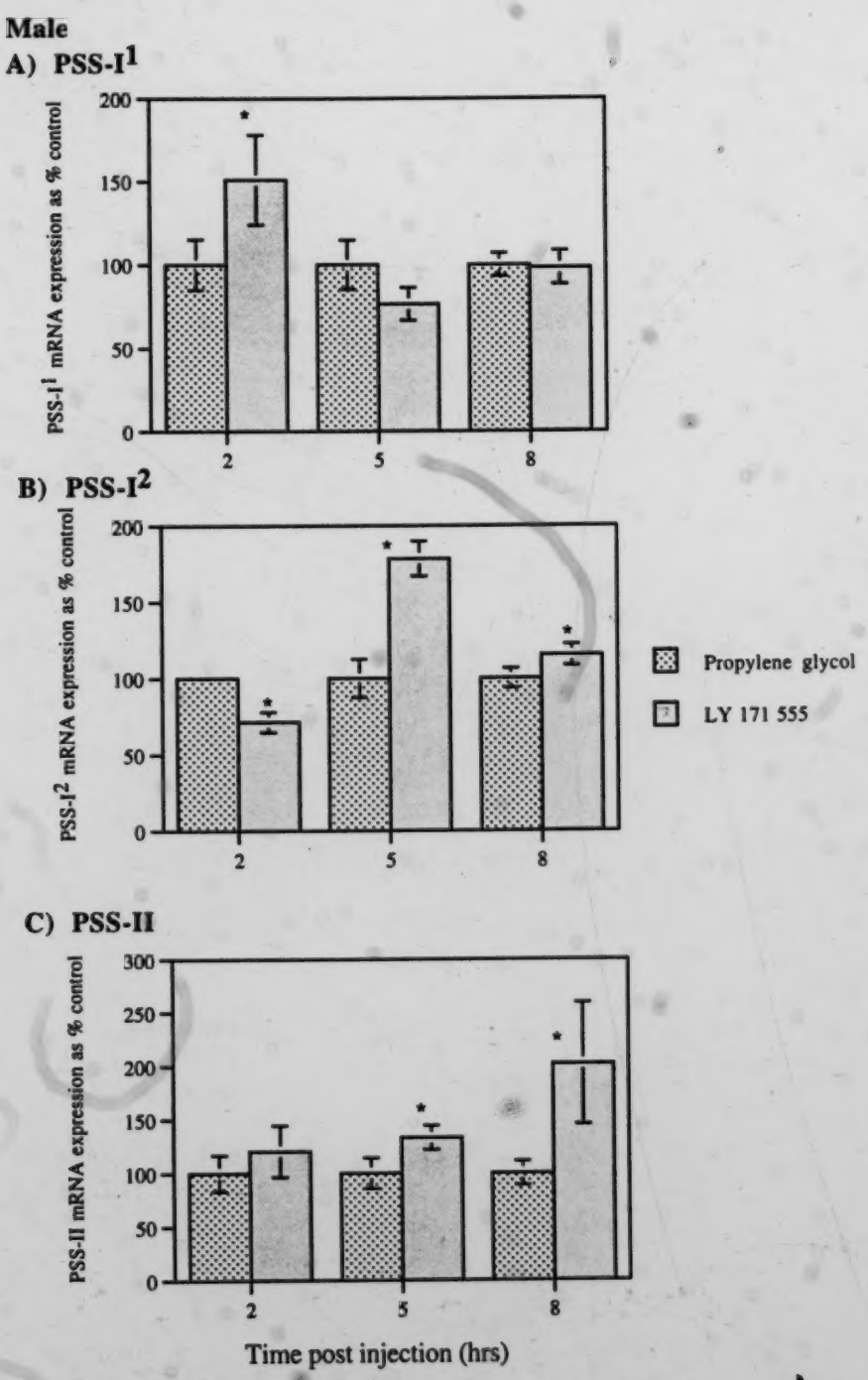

Figure 3-22: The effects of LY 171 555, a D_2 agonist, on A) PSS-I^1 B) PSS-I^2 and C) PSS-II mRNA expression in the forebrain of sexually recrudescent male goldfish; GSI=2.9%+/-0.26SEM. (*) shows a significant difference from control ($P<0.05$; n=4).

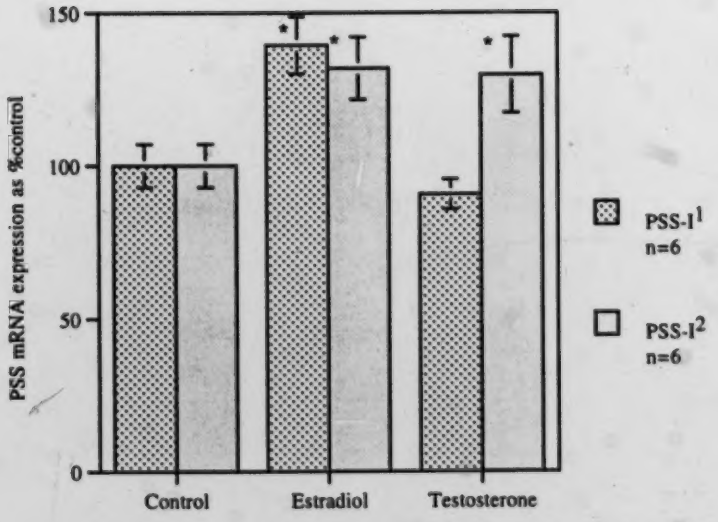

Figure 3-23: The effect of 5 day exposure to estradiol, testosterone or no steroid (control) pellets on PSS-I^1 and PSS-I^2 mRNA expression in the telencephalic-preoptic area of sexually regressed female goldfish, GSI=1.15%+/-0.11. (*) represents a significant difference from control implant ($P<0.05$).

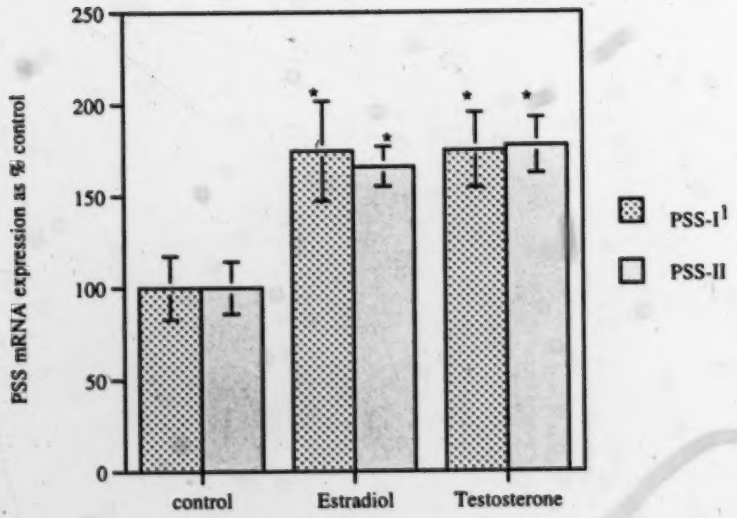

Figure 3-24: The effect of 5 day exposure to estradiol, testosterone and blank (control) pellets on PSS-I[1] and PSS-II mRNA in the hypothalamus of sexually regressed female goldfish, GSI=1.15%+/-0.11. (*) shows a significant difference from control ($P<0.05$; n=6).

Chapter 4

Discussion

Phylogenetic Origins of Goldfish Somatostatin Genes.

SS is unique in teleosts compared to other vertebrates because it has two distinct preprohormones encoding SS-14 and a longer variant. Our search for two SS cDNAs lead to the discovery of a third preprohormone encoding a unique form of SS-14 with a substitution from glycine to proline at position 2 of the mature peptide. This variant ([Pro^2]SS-14) has been previously isolated from Russian sturgeon pituitaries (Nishi *et al.* 1995). This variant cDNA, PSS-I^2, is found in the presence of SS-14 (PSS-I^1) and a unique goldfish SS-28 (PSS-II) cDNA sequence.

The phylogenetic tree of coding regions for the known PSS cDNA sequences displays a distinct commonality between the cDNA's encoding a mature SS-14 peptide and a distinct group of peptides encoding larger forms. The coding region of the cDNA for PSS-I^2 is not grouped with either of the other two goldfish PSS cDNAs, instead it is most closely related to frog[Pro^2, Met^{13}]SS-14 peptide encoding cDNA (Figure 3-2). Previous literature also suggests a common ancestral origin for frog [Pro^2, Met^{13}]SS-14 and cortistatin in the rat (Conlon *et al.* 1997). Goldfish PSS-I^2 and rat cortistatin have a 31% sequence homology, with both peptides containing a processing site for a 29 AA peptide. [Pro^2, Met^{13}]SS-14 cDNA from frog has 50% homology with rat cortistatin. Between frog [Pro^2, Met^{13}]SS-14 cDNA and goldfish PSS-I^2 there is 41% sequence homology. Because of the similarity of cortistatin and [Pro^2]SS-14 to [Pro^2, Met^{13}]SS-14, these two peptides likely diverged from the same ancestral gene.

Cortistatin is a 14 AA peptide sharing 12 of its 14 residues with [Pro^2]SS-14 and 13 of the 14 with [Pro^2, Met^{13}]SS-14, as well as all three having the cyclical structure

created by the cysteine residues. There is also a possible Lys-Arg processing site for a mature 29 -residue peptide (rCST29) (de Lecea *et al.* 1997a). The human putative cortistatin peptide has an arginine for lysine substitution, compared to the rat and mouse products, and is N-terminally extended by 3 amino acids (de Lecea *et al.* 1997b). At the C-terminal portion of the coded protein, at least two different sizes of mature peptides with 29 and 17 amino acid residues, respectively may be produced in the human. Cortistatin-17 binds to all human SSTR subtypes in almost the same manner as rat cortistatin-14. It also inhibits cAMP production induced by forskolin through SSTRs. Cortistatin is known to bind to SSTR in GH_4 pituitary cells and is expressed in a distinct subset of interneurons in the rat cerebral cortex and hippocampus (de Lecea *et al.* 1996). Cortistatin is also found in the hypothalamus and pituitary of the rat (Tominaga *et al.* 1992). The function of cortistatin in the hypothalamus and its role in the release of hypophysiotrophic hormones is yet to be investigated in the rat or human with little physiological information known about the function of [Pro^2, Met^{13}]SS-14 in frogs. In frogs, [Pro^2, Met^{13}]SS-14 is found in the ventral hypothalamic nucleus and the anterior preoptic area and is able to inhibit the release of GH from the adenohypophysial cells (Jeandel et al 1998). The unique actions of cortistatin in the cortex of mammals supports the hypothesis of [Pro^2]SS-14 as a unique neurohormone in the brain of lower vertebrates with actions unlike SS-14.

The deduced SS-28 peptide from PSS-II showed 5 AA differences from the SS-28 previously isolated from the intestinal tract of goldfish (Uesaka *et al.* 1995). This suggests one ancestral gene encoding SS-28 which duplicated early in teleost evolution producing two genes encoding unique SS-28 peptides in the goldfish. The common goldfish *Carassius auratus* and the closely related common carp *Cyprinus carpio* are both tetraploid, as they have ≈100 chromosomes, twice the number of most other cyprinid fishes (Ohno *et al.* 1968). Therefore any DNA or protein sequences in the goldfish should be evaluated remembering that duplicate loci with divergent sequences may exist. These may be copies of the same ancestral gene.

Distribution

The unique distribution of all three PSS mRNA in the digestive and renal systems confirms that transcription of PSS-I^1, PSS-I^2, and PSS-II mRNAs are not restricted to the brain. Biological actions of peripheral SS in vertebrates include the inhibition of pancreatic and gastrointestinal tract hormone secretion (Gerich 1990), influencing intestinal and blood ion transport systems, as well as affecting the levels of some plasma metabolites (Plisetskay 1980; Stewart *et al.* 1978). From our distribution studies PSS-I^1 and PSS-II are detected in the liver. SS has previously been described in islet cells of coho salmon (Plisetskaya *et al.* 1986). In goldfish, however, islet cells are not separate from the liver. Somatostatin-binding sites in trout hepatic membrane preparations have also been characterized (Pesek and Sheridan 1996). SS stimulates hepatic lipolysis in rainbow trout, with fasting enhancing hepatic sensitivity to SS-14 binding. PSS-I^1 and PSS-II are the forms detected in the anterior portion of the GI-tract. Uesaka *et al.* (1995) isolated somatostatin in the intestine of goldfish. Endogenous somatostatin (eSS-25II) activated net Na^+, Cl^- and water fluxes across the seawater eel intestine (Uesaka *et al.* 1994). SS-14 and sSS-25 are also important regulators of carbohydrate and lipid metabolism in rainbow trout, and modulation of metabolic activity by these peptides may be accomplished, in part, by alterations in insulin and glucagon levels circulating in the plasma (Eilertson and Sheridan 1993). It appears PSS-I^2 is the only SS present in the kidney, suggesting another unique function for the SS-14 variant.

Our results from pituitary cell mRNA expression confirm the synthesis of PSS-I^1 mRNA in the pituitary itself. The other two forms are not found in pituitary cells. This is the first study to localize PSS-I^1 mRNA in the pituitary of vertebrates. SS-14 in the pituitary may act as a local paracrine control of GH secretion, or of other hormones such as TSH, prolactin (PRL) or MSH. Because cultured pituitary cells were used nerve fibers in

the neurohypophysial stock cannot be considered the source. An assumptions is made that SS mRNA is not secreted from the hypothalamus and taken up by pituitary cells. All five SSTR are present in the anterior pituitary of rats. SSTR 1-4 are in the intermediate lobe and 2 and 3 are in the neural lobe. SSTR4 and SSTR5 are more prevalent on GH cells. In goldfish only immunoreactive SS has been studied with no literature on goldfish SSTR.

The distributions of the three SS mRNAs in the brain are distinct suggesting a possible functional difference between them. PSS-I^1 is widely distributed throughout the six regions of the brain tested, excluding the olfactory bulbs, suggesting possible actions as a neurotransmitter or neuromodulator. PSS-I^2 is also widely distributed throughout the six regions of the brain, including the olfactory bulbs, with lower concentrations in the optic tectum-thalamus and posterior brain, detectable only after PCR amplification. PSS-II, however, is localized to the hypothalamus and optic tectum-thalamus. The brain distribution of SS-14 has previously been examined through immunohistochemistry (Kah *et al.* 1982), but the specificity for the antibody between the different forms is unknown. The results on brain distribution of PSS mRNAs are in agreement with previous studies showing SS immunoreactive perikarya in the nucleus preopticus periventricularis (NPP), the nucleus lateral tuberis (NLT), and the dorsomedial hypothalamus in rainbow trout (Dubois *et al.* 1979), Gambusia sp., and goldfish (Kah *et al.* 1982); however, no staining was previously found in the olfactory bulb. The NLT neurons are known to innervate the hypophysis (Peter *et al.* 1990). When lesions were done in the preoptic region of the goldfish by Fryer *et al.* (1981), pituitary GH cells were activated as seen through ultrastructural studies. Cook and Peter (1983) were also able to show an increase in serum GH levels and greater growth after lesioning the preoptic region. Our findings support this by demonstrating the synthesis of PSS-I^1 and PSS-I^2 in the preoptic area. GHRH is also found in the NPP of the preoptic area (Rao *et al.* 1996), along with DA (Kah *et al.* 1986), galanin (Prasada *et al* .1996; Cornbrooks and Parsons 1991; Olivereau and Olivereau 1991) and GnRH (Lin and Peter 1996; 1997) providing the possibility of indirect effects on GH

secretion through interactions of somatostatinergic neurons with other neurons involved in the regulation of GH release.

Distribution studies of PSS-I^2 revealed a wide distribution through the whole brain. The full physiological effects of this novel peptide are unknown at this time, but the wide spread distribution seen though Northern analysis and RT-PCR suggests a neuromodulator or neurotransmitter role. As previously mentioned, cortistatin is found throughout the brain including the hypothalamus and pituitary of the rat, showing further parallels between the two peptides. Staining of cortistatin in the pituitary is believed to be in somatotrophs or gonadotrophs, but definitely not corticotrophs (Tominaga *et al.* 1992), suggesting a further similarity between PSS-I^2 and cortistatin.

In this study, all three PSS forms were located in the hypothalamus. This is in agreement with previous immunohistological studies describing SS cell bodies in the periventricular nucleus of the hypothalamus (Kah *et al.* 1982), with SS nerve terminals frequently identified close to the somatotrophs in the pituitary (Olivereau *et al.* 1984 a&b). Stimulators of GH secretion in goldfish are also localized in the hypothalamic region. NPY is known to be colocalized with SS neurons with in the hypothalamus (Pickavance *et al.* 1992) and therefore may also be involved in decreasing SS gene expression to increase GH secretion. In the brain of the euryhaline, viviparous teleost *Poecilia latipinna* (green molly) SS immunoreactive (SS-IR) neurons were numerous throughout the periventricular areas of the diencephalon. In the brain of common carp additional groups of SS-IR neurons, extending from the ventral telencephalon into the dorsal telencephalon pars centralis, also contained neuropeptide Y (NPY)-, peptide YY (PYY)-, and NPY flanking peptide (PSW)-like-immnoreactivity (IR) (Batten *et al.* 1990).

PSS-I^2 is uniquely found in the olfactory bulbs. Other neurohormones involved in growth hormone secretion that are located in the olfactory bulbs include TRH, sGnRH, and CCK. In the tilapia hybrid (*Oreochromis niloticus* x *Oreochromis aureus*) thyrotropin-releasing hormone (TRH) had no effect on GH secretion from perifused pituitary

fragments, but increased plasma GH levels, as did bovine thyroid stimulating hormone (bTSH) *in vivo* (Melamed *et al.* 1995). Regulation of GH by TRH therefore may occur at the level of SS expression or secretion. TRH-IR in the brain of juvenile chinook salmon is also located in the preoptic region of the diencephalon, and in the supracommissural nucleus of the ventral telencephalon (Matz and Takahashi 1994). Salmon GnRH-IR cell bodies have also been localized in the ventral telencephalon, the preoptic area, and the hypothalamus (Kim *et al.* 1995).

Biological Activity

Testing the biological activity of [Pro^2]SS-14 demonstrated the functional similarity between SS-14 and [Pro^2]SS-14 on the inhibition of release of GH from pituitary fragments. [Pro^2]SS-14 is equipotent to SS-14 in the dose response curves for the inhibition of GH secretion, suggesting a similar role for SS-14 and [Pro^2]SS-14 in GH secretion. A preliminary study on [Pro^2]SS-14 pituitary GH-inhibition in rainbow trout also displayed a dose response curve similar to SS-14 (Nishi *et al.* 1995). Binding studies on [Pro^2, Met^{13}]SS-14 in the frog pituitary, however, show [Pro^2, Met^{13}]SS-14 to be more potent then SS-14 when competing with the radioligand (Jeandel *et al.* 1998). Since the frog variant is closer in phylogenetic origin to [Pro^2]SS-14 than SS-14, [Pro^2]SS-14 may play a more important role in GH regulation than SS-14. In the goldfish this difference, however, is not due to differences in potency of the two peptides on GH inhibition, but might instead occur at the level of gene expression, translation or metabolism. Further experiments should be done to investigate the similarities and differences between the gene expression and translation systems for the two SS-14 peptides in the goldfish.

In the frog the two peptides, [Pro^2, Met^{13}]SS-14 and SS-14, are known to inhibit GRF-stimulated GH secretion but do not alter basal GH secretion (Jeandel *et al.* 1998). SS-14 also has no effect on basal GH secretion from ovine pituitary cells (Wu *et al.* 1996).

SS-14 in the rat (Brazeau *et al.* 1973) and goldfish (Marchant *et al.* 1987), however, has been shown to inhibit both basal and stimulated GH secretion. The dose response experiment from the present study displays an inhibition of basal GH secretion by [Pro^2]SS-14, therefore the two goldfish SS-14 peptides can be hypothesised to work through the same receptor system. This receptor system, however, appears to differ from the ovine and frog receptors in its intracellular inhibition of GH secretion.

[Pro^2]SS-14 was also able to block two GH stimulating peptides known to act via different second messenger systems (Chang *et al.* 1994) indicating that DA D_1receptor- and sGnRH-stimulated GH release is also subject to the inhibitory control of [Pro^2]SS-14. In the goldfish, the intracellular signal transduction mechanisms mediating the effects of two stimulatory regulators of GH release, GnRH and dopamine, have been studied (for review see: Chang *et al.* 1994; 1996). GnRH actions on GH release are mediated by activation of protein kinase C (PKC) and extracellular calcium entry through high voltage-activated, voltage-sensitive calcium channels (VSCC). In contrast, dopamine D_1-receptor actions on GH release are mediated primarily by cyclic (c)AMP-dependent mechanisms, but not by PKC. This allows for the additive effect seen on GH secretion when the two peptides are used in consort. Our experiments with SS-14 and [Pro^2]SS-14 show similar inhibition of the two GH stimulating pathways, suggesting again that the same receptors are used in inhibiting GH secretion by the two peptides.

In mammals a rebound of GH release is observed after SS treatment, which can be attributed to the inhibition of SS on GH release without the concurrent inhibition of GH synthesis (Cowan *et al.* 1983). This GH rebound is believed to be a part of the regulatory mechanisms responsible for the episodic GH release in the rat (Kraicer *et al.* 1986). In mammals the main outcome of SSTR binding by SS is a decrease in Ca^{2+} influx and a decrease in intracellular free- Ca^{2+} together causing a decrease in GH secretion (for review see: Harvey 1996). An indirect effect of SS on GH synthesis is seen by the inhibition of GHRH-stimulatory actions on GH through the antagonism of adenylate cyclase activity.

Otherwise, no direct effects on GH synthesis have been detected in both mammals and teleosts (Harvey 1996; Yada and Hirano 1992; Melamed *et al.* 1996). In this study, the rebound of GH release after SS-14 or [Pro^2]SS-14 was not seen and has not been seen in other teleost species in *in vitro* studies(Wong *et al.* 1998; Wong *et al.* 1993; Lin *et al.* 1993, Marchant *et al.* 1987). A rebound however has been seen with SS-14 in *in vivo* studies (Cook and Peter 1984).

The remainder of this study involves the comparison of expression of all three known PSS mRNA. The effects of PSS-I^1 and PSS-I^2 on GH have been discussed above, however, the effect of goldfish brain SS-28 on GH is unknown. In previous studies by Marchant *et al.* (1987) three longer forms of the SS peptide were studied in relation to their effect on GH secretion from pituitary fragments, mammalian SS-28, salmon SS-25 (sSS-25) and catfish SS-22 (cSS-22). The results showed no difference in potency for the inhibition of GH between SS-28 and SS-14 from mammals. sSS-25 and cSS-22, however, showed no significant inhibition of GH secretion. cSS-22 varies from mammalian SS-28 at 7 of 14 amino acids in the carboxyl terminus alone. sSS-25, however, varies at only two amino acids in the carboxyl terminus (see Appendix II). These two amino acid changes are also present in the deduced sequence from our goldfish brain PSS-II cDNA. The importance of PSS-II in the forebrain, and possibly GH secretion, is unknown but studies of gene expression will aid in the discovery of its physiological relevance.

Seasonal Variation

In this study we have shown that PSS-I^2 and PSS-II mRNA expression levels are highest for both males and females in late July in sexually regressed fish, and lowest in April in sexually mature fish, with December as an important time of transition in PSS-I^2 gene expression (Figure 3-12) due to the significant differences seen between the males and females at this time. The seasonal variations in PSS-I^2 and PSS-II mRNA expression

levels are inverse to GH serum levels previously measured by Marchant and Peter (1986). Previous studies by Marchant and Peter (1989a) have shown SS-IR levels in the serum, as well as SS-IR in the telencephalon and hypothalamic areas of the forebrain, to change in a manner inverse to serum GH levels (Marchant and Peter 1989b). Our results, therefore, expand on Marchant's work to show that SS is inversly related to serum GH levels as expected of an inhibitor. SS is controlled at the level of transcription to express high levels of SS in sexually regressed fish and lower levels in sexually recrudescent to mature fish. This is also the first time seasonal SS variation can be more specifically identified as a variation in [Pro^2]SS-14 and possibly SS-28 synthesis. Whether the seasonal variation in serum GH levels identified by Marchant and Peter (1986) is the cause or effect of fluctuations in SS expression is as yet unknown. It is also not known whether SS antiserum used by Marchant and Peter (1989b) cross reacts with [Pro^2]SS-14.

No seasonal variation in PSS-I^1 mRNA was detected in the forebrain of males suggesting that SS-14 has little, to no, effect on the seasonal changes in GH levels. In females there is a seasonal variation, with higher PSS-I^1 levels in sexually mature and maturing fish and lower levels in sexually regressed fish. This pattern of PSS-I^1 mRNA expression suggests that PSS-I^1 may be reacting to the high GH levels in female fish, but is not involved directly in controlling the seasonal GH cycle. These distinct differences between males and females in PSS-I^1 mRNA expression implicate sex steroids in the modulation of PSS-I^1 mRNA expression in the forebrain. From this seasonal data it could be suggested that sex steroids, possibly estradiol, produced in maturing females have a stimulatory role in PSS-I^1 mRNA expression, while sex steroids in males have no effects on PSS-I^1 mRNA expression. This hypothesis will be discussed more completely later in the discussion.

A novel finding of this study is the differential PSS mRNA expression between males and females in a mature state. Unfortunately, Marchant and Peter (1989b) studied the SS-IR of female fish only, and no seasonal differences in serum GH levels were found

between males and females (Marchant and Peter 1986). Our results show that all three PSS mRNA have significantly higher levels of expression in sexually mature female fish than male fish. This is opposite of the rat where male rats have higher SS mRNA synthesis compared to female rats; with the lower expression of SS mRNA in females being able to be stimulated with an injection of testosterone (Chowen *et al.* 1996). This difference suggests a species specific control of SS gene expression by sex steroids.

In our experiment, goldfish forebrain samples were extracted in December as a representation of PSS mRNA expression in sexually recrudescent fish. Results showed a significant difference between males and females in PSS-I^2 mRNA expression. PSS-I^2 mRNA expression is lower in female fish than males in December with female PSS-I^2 mRNA expression levels already down at a level similar to females in April. December is a period of gonadal growth in the goldfish and GH serum levels are just starting to rise. The female samples at this time are not significantly different from the April samples suggesting the modulators of PSS-I^1 and PSS-I^2 mRNA expression acting in the mature fish are already starting to influence female fish as early as December.

Sex Steroids Effects on PSS mRNA Expression in the Forebrain of Goldfish

In this study, the effects of estradiol (E_2) and testosterone (T) on PSS mRNA expression in the telencephalic-preoptic area and hypothalamus were tested in sexually regressed female fish. In the telencephalic-preoptic area E_2 increased the expression of both PSS-I^1 and PSS-I^2 mRNA. Effects of T, however, were only seen in the stimulation of PSS-I^2 mRNA expression, not PSS-I^1. As mentioned above, the telencephalic-preoptic area has been shown to be important in the inhibition of GH secretion from the pituitary. It was therefore important to test both these known GH-inhibiting peptides found in this region in order to determine the importance of sex steroids in the seasonal variation of somatostatin gene expression and therefore GH secretion. In the hypothalamus PSS-I^1

and PSS-II mRNA expression increased after E_2 and T implantation. PSS-II is only expressed strongly in the hypothalamus.

In mammals, gonadal steroid hormones have been shown to affect both hypothalamic SS content and synthesis (reviewed by Estupina *et al.* 1996). In addition, hypothalamic SS content and SS mRNA levels in the hypothalamus are generally reduced following gonadectomy. Levels of SS can be restored by administration of steroid hormones. In the rat estrous cycle, SS release and hypothalamic content exhibit concomitant fluctuations with plasma levels of gonadal steroids.

The effects of the i.p. implantation of the steroid pellets can be either direct though steroid receptors on SS neurons, or indirect through neuromodulators or pituitary hormones. The ability of E_2 to increase PSS mRNA may explain the higher levels of PSS-I^1, PSS-I^2 and PSS-II mRNA levels found in mature females in the seasonal study. The effects of sex steroids on PSS-I^1 and PSS-I^2 mRNA expression, however, do not appear to be responsible for the main seasonal variation in PSS mRNA expression but are only responsible for the difference seen between sexually mature male and female fish. It is interesting to note a recent experiment in rainbow trout which found plasma SS levels to decrease with exposure to E_2 treatment (Holloway and Leatherland 1997). A decrease in plasma SS levels in fish has also been shown by Marchant and Peter (1989b) in goldfish during high gonadal steroid levels. As mentioned in the introduction, however, peripheral plasma levels are known to vary inverse to brain levels of SS and that the importance of serum SS in the control of GH is unknown. Holloway and Leatherland (1997) also reported that, after two weeks i.p. implantation of E_2, SS-14 had no inhibitory effects on GH secretion from pituitary glands *in vitro* (Holloway and Leatherland 1997). This suggests the regulation of SS on GH is controlled at a post-transcriptional level, and possibly at the level of SSTR receptor. Therefore a rise in the expression of SS gene expression does not infer a decrease in GH secretion in the mature goldfish.

Indirect effects, however, may also be seen through other neuromodulators such as DA. As dopaminergic neurons are known to be present in the preoptic region, and results discussed below indicate that DA influences SS gene expression, E_2 receptors present on DA synthesizing neurons in rainbow trout (Linard *et al.* 1996) provide a basis for indirect actions of E_2 on PSS expression. The effect of the steroids may also be indirect through the feedback of growth hormone which has been shown to increase after sex steroid implantation (Trudeau *et al.* 1996). More specifically, studies have shown E_2 to increase serum GH levels in regressed fish (Zou *et al.* 1997). The rise in PSS mRNA expression in the telencephalic-preoptic area and hypothalamus could be the effect of GH feedback from the increased serum GH levels. GH negative feedback on SS in the rat is supported by the presence of GH receptors on SS neurons in the brain (Burton *et al.* 1992).

As mentioned earlier PSS-I^1 mRNA had an increase in expression in sexually mature female fish with no difference between sexually regressed and mature males. This suggests that the main regulation of seasonal changes in PSS-I^1 mRNA is by the female sex steroids. By using steroid implantation in female fish we have shown PSS-I^1 in the telencephalic-preoptic area to increase with estradiol implantation but not testosterone implantation. This result also suggests that PSS-I^1 mRNA in the telencephalic-preoptic area of the forebrain may be more predominant in sexually mature female fish than PSS-I^1 mRNA in the hypothalamus which is increased by both E_2 and T. It also suggests that the androgen receptor distribution and possibly the distribution of aromatase, converting T to E_2, are unique for each brain region. Immunolocalization of aromatase and androgen receptors in the brain of goldfish defined the highest concentration of both T binding receptors in the reproductive centers: medial and ventral telencephalon, preoptic area and hypothalamic periventricular nuclei (Gelinas and Callard 1997). Whether the aromatase and androgen immunoreactivity was on PSS expressing cells is unknown.

sGnRH Effects on PSS mRNA Expression in the Forebrain of Goldfish.

sGnRH is a major stimulator of GH secretion in goldfish (Peng and Peter 1997). At five hours post-injection of sGnRH, the level of expression of PSS-II mRNA was increased; however, no changes were found in the levels of expression of PSS-I^1 and PSS-I^2 mRNA. As the function of this SS-28 peptide is not known in teleosts, the significance of this interaction of sGnRH on PSS-II mRNA expression is not known. Although additional work needs to be done to more thoroughly demonstrate the lack of effect of sGnRH on expression of PSS-I^1 and PSS-I^2 mRNA, the present results indicate that there is no interaction between GnRH and SS-14 or [Pro2]SS-14.

Dopaminergic Effects on PSS mRNA Expression in the Forebrain of Goldfish.

The possible actions of dopamine on expression of PSS mRNA were investigated first by injection of, α-MPT, a blocker of tyrosine hydroxylase enzyme activity responsible for the conversion of tyrosine to dihydroxyphenylalanine (L-dopa) and subsequent synthesis of catecholamine neurotransmitters. Our results show a decrease in PSS-I^2 and PSS-II mRNA levels with no change in PSS-I^1 mRNA levels following injection of α-MPT, indicating an important input of dopaminergic neurons on SS neurons.

In the goldfish the anterior pituitary is under the direct innervation of a dopaminergic preoptic-hypophysial pathway, and DA nerve terminals have been identified in the close proximity of somatotrophs (Kah *et al.* 1987). These immunohistochemical observations provide an anatomical basis for the involvement of DA in GH regulation. DA and its non-selective agonist apomorphine are stimulatory to GH release in the goldfish, both *in vivo* (Chang *et al.* 1985) and *in vitro* (Peter *et al.* 1990). Wong *et al.* (1992) further demonstrated that DA directly stimulates GH release via D_1 receptors. Administration of α-MPT has previously been shown to increase GTH levels at 4 hr post injection (Trudeau

et al. 1993), decrease pituitary DA concentrations in sexually recrudescent male goldfish at 8 hr (Sloley *et al.* 1991), and cause a significant decrease in serum GH concentrations at 2 hr and 6 hr post injection. In addition to disrupting the direct actions of DA on somatotrophs, α-MPT may be acting at the level of brain, by releasing a tonic stimulatory influence of DA or NE on SS neurons expressing PSS-I^2 and PSS-II mRNA. Yet another explanation may be that through long-loop feedback GH itself may influence expression of PSS-I^2 and PSS-II mRNA. NE has previously been seen to decrease GH serum levels after an i.p injection but not after an i.c.v. injection, suggesting that, NE has no direct effects on the brain in controlling GH (Chang *et al.* 1985). However, at five hours following α-MPT treatment serum GH levels are decreased and therefore the SS neuronal system may be responding to the decrease in GH by decreasing PSS expression in the forebrain.

To further explore the actions of DA, i.p. injections of apomorphine, a non-selective DA agonist, were administered and forebrain samples were collected five hours after injection. The actions of DA on GH release at the level of the pituitary are mediated through DA D_1 receptors (Chang *et al.* 1990; Wong *et al.* 1992). The dopamine receptors on somatotrophs exhibit pharmacological properties characteristic of mammalian D_1 receptors (Wong *et al.* 1993b). Apomorphine is a non-selective DA agonist that can cross the blood-brain barrier and it has been shown to increase serum GH levels from 4 to 6 hours post injection (Chang *et al.* 1985). Apomorphine is known to directly stimulate GH release by acting on D_1 receptors on somatotrophs from the pituitary. The results of the present study show a decrease in expression of all three PSS forms. Because apomorphine can cross the blood-brain barrier it may be directly inhibiting PSS mRNA expression, thereby contributing to the increase in GH secretion by decreasing the actions of [Pro^2]SS-14.

Receptor specificity of DA action on PSS mRNA expression in the brain was then tested with the use of D_1 and D_2 agonists, SKF 38393 and LY 171,555 respectively,

which have been previously characterized in goldfish. D_1 receptor agonist, SKF 38393, stimulates GH release from perifused goldfish pituitary fragments, dispersed cells and isolated somatotrophs, showing direct effects (Wong *et al.* 1992; Wong *et al.* 1993b). Seasonal variations in the responses to DA agonists were found. Results from sexually regressed and recrudescent, SKF 38393 treated, fish are presented as mixed males and females because no significant differences were seen between the two sexes. Sexually regressed fish injected i.p. with SKF 38393 and sampled 5 hr post injection showed a decrease in the three PSS mRNA in the forebrain. These results are similar to those of apomorphine in regressed fish previously described. On the basis of the present results, it can be hypothesized that D_1 receptors also influence GH release by inhibiting PSS expression in regressed fish. SS immunoreactive cells in the preoptic area (Kah *et al.* 1982) are located in close proximity to the dopaminergic preoptico-hypophysial pathway therefore local DA action on PSS expression is physiologically possible.

In sexually recrudescent fish a change occurred in PSS-I^2 mRNA response to SKF 38393. Regressed fish show a decrease in PSS-I^2 expression due to SKF 38393, whereas sexually recrudescent fish had an increase in PSS-I^2 mRNA expression. This suggests that the D_1 receptor mediated action of DA on PSS-I^2 gene expression could be sensitive to sex steroid levels. Double staining studies showed consistently that most estrogen receptor-positive cells located ventral to the large extensions of the preoptic recess are also tyrosine hydroxylase-positive (Linard *et al.* 1996), providing an anatomical basis for DA-mediated sex steroid feedback action.

The DA D_1 mediated GH response can be blocked by the D_1 antagonists SCH 23390 and SKF 83566 (Wong *et al.* 1992). In our experiments, SCH 23390 was injected i.p. and forebrain PSS mRNA measured. These results showed the opposite effects of SKF 38393 administered in a parallel experiment. Antagonist results are only available for sexually recrudescent fish, but it confirms the unique rise in PSS-I^2 mRNA in the forebrain post SKF 38393. The DA D_1 mediated stimulation of GH release decreases as fish

progress through gonadal recrudescence and enter a mature sexual state (Wong *et al.* 1993b). Although this is thought to be a direct effect on somatotrophs, the present results demonstrate that a DA D_1 mediated increase in PSS-I^2 mRNA expression may also be a part of the seasonal changes in DA actions. Further studies at the level of the brain are needed to support this hypothesis; however, the present results clearly demonstrate that D_1 receptors are instrumental in the control of PSS mRNA expression as well as pituitary GH secretion.

In contrast to the action of DA D_1 agonists, the D_2 agonist LY 171,555 is not effective in stimulating GH secretion (Wong *et al.* 1993b). LY 171,555, otherwise known as quinpirole dihydrochloride, is a D_2 agonist which decreases cAMP synthesis in pituitary cells of rat (Meunier and Labrie 1982; Borgundvaag and George 1985). In the present study the effects of D_2 receptor activation on PSS mRNA expression were tested using LY 171,555 and the D_2 antagonist pimozide (PIM). In early recrudescence, LY 171,555 is seen to increase PSS-I^2 mRNA expression. Moreover, sexually mature fish injected with LY 171,555, also had a significant increase in expression of PSS-I^2 mRNA. When the antagonist PIM was injected no decrease in PSS-I^2 mRNA expression was seen. This suggests that there is no tonic stimulation of neurons expressing PSS-I^2 mRNA by D_2 receptors. The increase of PSS-I^2 mRNA expression due to LY 171,555 injection in recrudescent to mature fish is the same as the action seen in recrudescent to mature fish in response to D_1 agonist, SKF 38393, injection. This result suggests that DA receptor selectivity in neurons expressing PSS-I^2 mRNA may not be important. Both receptors appear to be able to regulate the gene expression of PSS-I^2 in the same manner. Results from the timecourse experiment mirrored previous five hour responses, with no response at two hours. After eight hours the response decreased almost back to control values. For SKF 38393 it fell significantly below control values.

At 5 hr post injection of LY 171,555 expression of both PSS-I^1 and PSS-II mRNA, in early recrudescent fish, show a decrease similar to their response to the D1

agonist, SKF 38393. Antagonists to both D_1 and D_2 receptors were able to significantly increase expression levels of PSS-I^1 and PSS-II suggesting the presence of a tonic inhibitory control by D_1 and D_2 receptors. In response to D_2 receptor stimulation in mature fish, PSS-I^1 shows no change from control at five hours, but an increase is seen at two hours. PSS-II mRNA and its peptide have an unknown function and therefore no conclusions towards its effects on GH can be drawn. A seasonal switch is seen in PSS-II mRNA expression in response to LY 171,555 injection and sexual differences are seen at 2 hrs post-injection of LY 171,555 in PSS-I^2 and PSS-II mRNA expression. Further investigation is needed to analyse these differences. The general increase in PSS-I^2 mRNA expression due to LY 171,555 injection suggests a link to the control of other systems, as well as GH secretion, with regulation of SS peptide effects on GH secretion controlled at a post-transcriptional level.

General Conclusions

In this study we set out to identify the somatostatin mRNA present in the brain of goldfish. Goldfish are a tetraploid animals so at least two copies of any one gene could be present in the goldfish. In teleosts, however, a complicating factor that has arisen for the somatostatin gene family is that the gene has been duplicated, producing two mRNA sequences to encode the two distinct peptide sequences. The timing of this gene duplication, in relation to tetraploidy, is unknown; however, it could be possible to have two copies of each somatostatin gene, encoding SS-14 and SS-28 peptides, in the genome. With our research we also discovered a third form of somatostatin, [Pro^2]SS-14. This form may be the result of a duplication event of either SS-14 or SS-28 but from analyzing its similarity with another known mammalian and frog genes, cortistatin and [Pro^2, Met^{13}]SS-14 respectively, it is most likely that was a unique gene already present when tetraploidy occurred. This information potentially complicates the SS system even more by

adding two more possible genes to the list of SS encoding genes. Furthermore, evidence has already been presented for the presence of two SS-28 genes; our cDNA sequence encodes a 28 AA long SS-like peptide different from the peptide sequence found in the intestine of goldfish by Uesaka *et al.* (1995).

This was also the first study to examine distibution of SS encoding genes throughout the goldfish. Distribution studies were focused primarily on the brain and pituitary, and six peripheral body tissues. We confirmed the localization of all three PSS genes in the hypothalamus, but only PSS-I^1 and PSS-I^2 were found in the preoptic area, suggesting a more direct role for SS-14 and [Pro^2]SS-14 in GH regulation. This is supported by lesioning studies of the preoptic region by Cook and Peter (1983). Because of the diffuse brain distribution of PSS-I^1, a neurotransmitter action of SS-14 is very likely in the goldfish.

Our novel finding of PSS-I^1 in the pituitary of goldfish opens possibilities for the direct action of SS on hypophysial hormone secretion through a paracrine action. High concentrations would not be necessary for a paracrine action in a relatively small population of cells. However, whether PSS mRNA expression is in somatotrophs or other cells in the pituitary is not known.

The seasonal effect of SS on GH hormone secretion involves the interaction of many other neurofactors and peripheral hormones in the body. SS neuronal system in the brain is surrounded by neurons containing other factors which are involved in the reproductive cycle of the goldfish. In this annual cycle, the pituitary hormones GTH-I and GTH-II increase and decrease in response to photoperiod, temperature and food availability in order to control the release of peripheral sex steroids from the maturing gonads. GnRH is the major stimulator and DA is the major inhibitor of GTH-II release. Many other neurofactors have also been implicated in the release of GTH-II in the goldfish but GnRH and DA are of interest due to their predominant role in the regulation of GH release as well. Our present study agrees with previous SS-IR studies by showing an inverse relationship

between serum GH levels and PSS-I^2 and PSS-II mRNA levels. The hypothesis that SS-14 acts mainly as a neurotransmitter/ neuromodulator in the brain is supported in this study by showing little, to no, seasonal variation in the forebrain.

Sex steroids most likely play a role in the regulation of PSS-I^1, PSS-I^2 and PSS-II mRNA expression in the forebrain of the goldfish during sexual maturity when sex differences are seen in PSS expression. From our implantation studies in female fish both E_2 and T have the ability to increase PSS-I^2 mRNA levels. It is assumed that T acts by aromatization to estradiol in this case. Further studies are needed on the effect of sex steroids on PSS mRNA in order to understand the sexual differences seen in mature fish. Our sex steroid studies were done on regressed fish to eliminate the confounding variable of endogenous steroid in the in *vivo* system used. Taken together it can be hypothesized that sex steroids have the ability to increase PSS mRNA, but most likely other factors such as DA mediate the overall seasonal variation seen in PSS-I^2 and PSS-II mRNA expression.

PSS mRNA expression was inhibited by DA in sexually regressed fish. DA is a major stimulator of GH secretion in the goldfish by actions on somatotrophs, and this study provides more evidence for an indirect action of DA on GH through SS, more specifically DA acts at the level of PSS mRNA expression. These effects, however, vary seasonally. The effects of the DA D_1 agonist most closely mimic the effects of the general DA agonist suggesting the presence of D_1 receptors on PSS mRNA producing cells. Further *in vivo* experiments will be required to confirm this hypothesis.

In conclusion, this study has demonstratedthe presence of three forms of SS mRNA and a distinct difference in their gene expression. While the three forms of SS may have a common ancestral gene, their functional significance in the brain of goldfish has diverged and specialized since that event. The gene encoding [Pro^2]SS-14 was uniquely regulated by the dopamine system and [Pro^2]SS-14 may have a prominent roll in regulating pituitary homone release, specifically GH. There is, however, physiological evidence presented for the regulation of all three forms of PSS by stimulators of GH secretion in the

brain of goldfish. While the physiological actions of SS-14 on GH release are well documented, the physiological function of goldfish brain SS-28 remains unknown.

Literature Cited

Abrahamsson PA, Wadstrom LB, Alumets J, Falkmer S, and Grimelius L 1986. Peptide-hormone- and serotonin-immunoreactive cells in normal and hyperplastic prostate glands. Pathol Res Pract 181:675-683.

Andrews PC, Pollock HG, Elliott WM, Youson JH, and Plisetskaya EM 1988. Isolation and characterization of a variant somatostatin-14 and two related somatostatins of 34 and 37 residues from lanprey (Petromyzon marinus). J. Bio. Chem. 263:15809-15814.

Anglade I, Pakdel F, Bailhache T, Petit F, Salbert G, Jego P, Valotaire Y,and Kah O 1994. Distribution of estrogen receptor-immunoreactive cells in the brain of the rainbow trout (Oncorhynchus mykiss). J Neuroendocrin 6: 573-583.

Baldino F Jr, Fitzpatrick-McElligott S, O'Kane TM, and Gozes I 1988. Hormonal regulation of somatostatin messenger RNA. Synapse 2(3):317-325.

Banks WA, and Kastin AJ 1985. Aluminum alters the permeability of the blood-brain barrier to some non-peptides. Neuropharmacology 24:407-412.

Battaglia G, Norman AB, Hess EJ, Creese I 1986a. Functional recovery of D1dopamine receptor-mediated stimulation of rat striatal adenylate cyclase activity following irreversible receptor modification by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ): Evidence for spare receptors. Neurosci Let 69: 290-295.

Battaglia G, Norman AB, Hess EJ, and Creese I 1986b. Forskolin potentiates the stimulation of rat striatal adenylate cyclase mediated by D1 dopamine receptors, guanine nucleotides, and sodium fluoride. J Neurochem 46: 1180-1185.

Batten TF, Cambre ML, Moons L, and Vandesande FJ 1990. Comparative distribution of neuropeptide-immunoreactive systems in the brain of the green molly, Poecilia latipinna. Comp Neurol 302(4):893-919.

Benoit R, Esch F, Bennett HP, Ling N, Ravazzola M, Orci L, and Mufson EJ 1990. Processing of prosomatostatin. Metabolism 39:22-25.

Benoit R, Ling N, and Esch F 1987. A new prosomatostatin-derived peptide reveals a pattern for prohormone cleavage at monobasic sites. Science 238:1126-1129.

Berelowitz M, Firestone S, and Frohman L 1981a. Effects of growth hormone excess and deficiency on hypothalamic somatostatin content and release and on tissue somatostatin distribution. Endocrinology 109:714-719.

Berelowitz M, Szabo M, Frohman LA, Firestone S, Chu L, and Hintz RL 1981b. Somatomedin-C mediates growth hormone negative feedback by effects on both the hypothalamus and the pituitary. Science 212:1279-1281.

Beaudet A, Greenspun D, Raelson J, and Tannenbaum GS 1995. Patterns of expression of SSTR1 and SSTR2 somatostatin recetpor subtypes in the hypothalamus of the adult rat: relationship to neuroendocrine function. Neuroscience 65:551-561.

Bissette G, and Myers B 1992. Somatostatin in Alzheimer's disease and depression. Life Sci 51(18):1389-1410.

Bjornsson B, Taranger GL, Hansen T, Stefansson SO, and Haux C 1994. The interrelation between photoperiod, growth hormone, and sexual maturation of adult atlantic salmon (Salmo salar). Gen Comp Endocrin. 93: 70-81.

Bluet-Pajot MT, Schaub C, Mounier F, Segalen A, Duhault J, and Kordon C 1980. Monoaminergic regulation of growth hormone in the rat. J Endocrin 86:387-396.

Borgundvaag B, and George SR 1985. Dopamine inhibition of anterior pituitary adenylate cyclase is mediated through the high-affinity state of the D2 receptor. Life Sci 37: 379-386.

Brakch N, Galanopoulou AS, Patel YC, Boileau G, and Seidah NG 1995. Comparative proteolytic processing of rat prosomatostatin by the convertases PC1, PC2, furin, PACE4 and PC5 in constitutive and regulated secretory pathways. FEBS Lett 362:143-146.

Brakch N, Rholam M, Nault C, Boileau G, and Cohen P 1991. Differential processing of hormone precursor: independent production of somatostatins 14 and 28 in transfected neuroblastoma 2A cells. FEBS 282:363-367.

Brazeau P Jr, Saucier R, and Husain SM 1972. Activities of hypothalamic HL-STH, of hypophyseal STH and of mimetric STH of plasma in adult and immature rats treated with enovid. Can J Physiol Pharmaco 50(10):967-970.

Brazeau P, Vale V, Burgus R, Ling N, Butcher M, Rivier J, and Guillemin R 1973. Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. Science 179: 77-79.

Bruhn TO, McFarlane MB, Deckey JE, and Jackson IM 1992. Analysis of pulsatile secretion of thyrotropin and growth hormone in the hypothyroid rat. Endocrinology 131:2615-2621.

Burton K, Kavigting EB, Clifton DK, and Steiner RA 1992. Growth hormone receptor messenger ribonucleic acid distribution in the adult male rat brain and its colocalization in hypothalamic somatostatin neurons. Endocrinology 131:958-963.

Cardinali DP, Esquifino AI, Arce A, Vara E, Ariznavarreta C, and Tresguerres JA 1994. Changes in serum growth hormone and prolactin levels, and in hypothalamic growth hormone-releasing hormone, thyrotropin-releasing hormone and somatostatin content, after superior cervical sympathectomy in rats. Neuroendocrin 59(1):42-48.

Caron P, Buscail L, Beckers A, Esteve JP, Igout A, Hennen G, and Susini C 1997. Expression of somatostatin receptor SST4 in human placenta and absence of octreotide effect on human placental growth hormone concentration during pregnancy. J Clin Endocrinol Metab 82:3771-3776.

Casanueva F, Betti R, Cocchi D, Zanardi P, and Motta EE 1981. A role for dopamine in growth hormone regulation in the dog. Endocrinology 108:1469-1475.

Chambers JW, and Brown GM 1976. Neurotransmitter regulation of growth hormone and ACTH in the rhesus monkey: Effects of biogenic amines. Endocrinology 98:420-428.

Chang JP and Peter RE 1984. Influence of norepinephrine and a-adrenergic mechanisms on gonadotropin secretion in female goldfish, Carassius auratus. Gen. Comp. Endocrin. 55:89-95.

Chang JP and Peter RE 1983b. Effects of pimozide and des Ghy10[D-Ala6]luteinizing hormone-releasing hormone ethylamide on serum gonadotropin concentrations, germinal vesicle migration, and ovulation in female goldfish, Carassius auratus. Gen. Comp. Endocrin. 52:30-37.

Chang JP and Peter RE 1983c. Effects of dopamine on gonadotropin release in female goldfish, Carassius auratus. Neuroendocrin. 36:351-357.

Chang JP, Cook AF, and Peter RE. 1983a. Influences of catecholamines on gonadotropin secretion in goldfish, Carassius auratus. Gen. Comp. Endocrin. 49:22-31.

Chang JP, Marchant TA, Cook AF, Nahorniak CS, and Peter RE 1985. Influences of catecholamines on growth hormone release in female goldfish, Carassius auratus. Neuroendocrin 40: 463-470.

Chang JP, Yu KL, Wong AOL, and Peter RE. 1990. Differential actions of dopamine receptor subtypes on gonadotropin and growth hormone release in vitro in goldfish. Neuroendocrin 51:664-674.

Chang JP, Van Goor F, Jobin RM, and Lo A. 1996. GnRH signaling in goldfish pituitary cells. Biological Signals 5:70-80.

Chang JP, Van Goor F, Wong AOL, Jobin RM, and Neuman CM 1994. Signal transduction pathways in GnRH- and dopamine D1-stimulated growth hormone secretion in the goldfish. Chin. J. Physiol. 37: 111-127.

Cheung S, Johnson JD, Moore KE, and Lookingland KJ 1997. Dopamine receptor-mediated regulation of expression of Fos and its related antigens (FRA) in somatostatin neurons in the hypothalamic periventricular nucleus. Brain Res 770:176-183.

Chihara K, Arimura A, and Schally AV 1979. Effect of intraventricular injection of dopamine, norepinephrine, acetylcholine, and 5-hydroxytrytamine on immunoreactive somatostatin release into rat hypophyseal portal blood. Endocrinology 104:1656-1662.

Chomczynski P and Sacchi N 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162(1):156-159.

Chowen JA, Garcia-Segura LM, Gonzalez-Parra S, and Argente J 1996. Sex steroid effects on the development and functioning of the growth hormone axis. Cell Mol Neurobiol 16(3):297-310.

Church GM and Gilbert W 1984. Genomic sequencing. Proc Natl Acad Sci U S A 81(7):1991-1995.

Conlon J M, Askensten U, Falkmer S, and Thim L 1988. Primary structure of somatostatins from the islet organ of the hagfish suggest an anomalous pathway of posttranslational processing of prosomatostatin-I. Endocrinology 122: 1855-1859.

Conlon JM, 1990. Somatostatin: aspects of molecular evolution. in Progress in comparative endocrinology, Wiley-liss, New York. pg 10-15.

Conlon JM, Davis MS, Falkmer S, and Thim L 1987. Structural charactization of peptides derived from prosomatostatins I and II from the pancreatic islets of two species of teleostean fish: the daddy sculpin and the flounder. Eur J Biochem 168:647-652.

Conlon JM, Tostivint H, and Vaudry H 1997. Somatostatin- and urotensin II-related peptides: molecular diversity and evolutionary perspectives. Regul Pept 69(2):95-103.

Cook AF, and Peter RE 1983. Effects of hypothalamic lesions on serum growth hormone levels and growth rates in goldfish, Carassius auratus. Gen Comp Endocrin 51(2):175-182.

Cook AF, and Peter RE 1984. The effects of somatostatin on serum growth hormone levels in the goldfish, Carassius auratus. J. Exp. Zoo. 237: 231-239.

Cornbrooks EB, and Parsons RL 1991. Sexually dimorphic distribution of a galanin-like peptide in the central nervous system of the teleost fish Poecilia latipinna. Comp Neurol 304(4):639-657.

Cowan NJ, Dobner PR, Fuchs EV, and Cleveland DW 1983. Expression of human alpha-tubulin genes: interspecies conservation of 3' untranslated regions. Mol Cell Biol 3(10):1738-1745

DeLean A, Munson PJ and Rodbard D 1978. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. Am J Physiol 235(2):E97-E102.

De Lecea L, Criado JR, Prospero-Garcia O, Gautvik KM, Schweitzer P, Danielson PE, Dunlop CL, Siggins GR, Henriksen SJ, and Sutcliffe JG. 1996. A cortical neuropeptide with neuronal depressant and sleep-modulating properties. Nature 381:242-245.

De Lecea L, del Rio JA, Criado JR, Alcantara S, Morales M, Danielson PE, Henriksen SJ, Soriano E, and Sutcliffe JG 1997a. Cortistatin is expressed in a distinct subset of cortical interneurons. J Neurosci 17(15):5868-5880.

De Lecea L, Ruiz-Lozano P, Danielson PE, Peelle-Kirley J, Foye PE, Frankel WN, and Sutcliffe JG 1997b. Cloning, mRNA expression, and chromosomal mapping of mouse and humanpreprocortistatin. Genomics 42(3):499-506.

Delitala G, Palermo M, Ross R, Coy D, Besser M, and Grossman A 1987. Dopaminergic and cholinergic influences on the gowth hormone response to growth hormone-releasing hormone in man. Neuroendocrin. 45:243-247.

Dournaud P, Gu YZ, Schonbrunn A, Mazella J, Tannenbaum GS, and Beaudet A 1996. Localization of the somatostatin receptor SST2A in rat brain using a specific anti-peptide antibody. J Neurosci 16(14):4468-4478.

Dubois MP, Billard R, Breton B, and Peter RE 1979. Comparative distribution of somatostatin, LH-RH, neurophysin, and alpha-endorphin in the rainbow trout: an immunocytological study. Gen Comp Endocrin 37(2):220-232.

Eden S, Bolle P, and Modigh K 1979. Monaminergic control of episodic growth hormone secretion in the rat: effects of reserpine, a-methyl-p-tyrosine, P-chlorophenyl alanine, and haloperidol. Endocrinology 105:523-529.

Eilertson CD, and Sheridan MA 1993. Differential effects of somatostatin-14 and somatostatin-25 on carbohydrate and lipid metabolism in rainbow trout Oncorhynchus mykiss. Gen Comp Endocrin 92(1):62-70.

Elazar Z, Siegel G, Fuchs S 1989. Association of two pertussis toxin-sensitive G-proteins with the D2-dopamine receptor from bovine striatum. EMBO J 8:2353-2357.

Elgort A, and Shields D 1994. Prosomatostatin processing in pituitary GH3 cells. Amer. society Biochem. Mol. biol. 269:30668-30675.

Enjalbert A, and Bockaert J 1983. Pharmacological characterization of the D2 dopamine recetpor negatively coupled with adenylate cyclase in rat anterior pituitary. Mol Pharmacol. 23:263-264.

Epelbaum J 1986. Somatostatin in the central nervous system: physiology and pathological modifications. Prog Neurobiol 27(1):63-100.

Epelbaum J, Willougyhby JO, Brazeau P, and Martin B 1977. Effects of brain lesions and hypothalamic deafferentation on somatostatin distribution in the rat brain. Endocrinology 101:1495-1502.

Estupina C, Pinter A, Belmar J, Astier H, and Arancibia S 1996. Variations in hypothalamic somatostatin release and content during the estrous cycle in the rat. Effects of ovariectomy and estrogen supplementation. Neuroendocrin 63(2):181-187.

Faria AC, Bekenstein LW, Booth RA Jr, Vaccaro VA, Asplin CM, Veldhuis JD, Thorner MO, and Evans WS 1992. Pulsatile growth hormone release in normal women during the menstrual cycle. Clin Endocrinol (Oxf) 36:591-596.

Fitzpatrick-Mcelligott S, Card JP, Lewis ME, and Baldino F. Jr. 1988. Neuronal localization of prosomatostatin mRNA in the rat brain with in situ hybridization histochemistry. J. Comp. Neurol. 273:558-572.

Florio T, Ventra C, Postiglione A, and Schettini G 1991. Age-related alterations of somatostatin gene expression in different rat brain areas. Brain Res 557:64-68.

Fodor M, Csaba Z, Epelbaum J, Vaudry H, and Jegou S 1998. Interrelations between hypothalamic somatostatin and proopiomelanocortin neurons. J Neuroendocrinol 10:75-78.

Fryer JN 1981. Hypothalamic lesions stimulating growth hormone cell activity in the goldfish. Cell Tissue Res 214(2):387-395.

Gelinas D, and Callard GV 1997. Immunolocalization of aromatase- and androgen receptor-positive neurons in the goldfish brain. Gen Comp Endocrin 106(2):155-168.

Gerich JE 1990. Role of somatostatin and its analogues in the pathogenesis and treatment of diabetes mellitus. Metabolism 39(9 Suppl 2):52-54.

Gil-Ad I, Laron Z, and Koch Y 1991. Effect of acute and chronic administration of clonidine on hypothalamic content of growth hormone-releasing hormone and somatostatin in the rat. J Endocrinol 131:381-385.

Gluschankof P, Gomez S, Morel A, and Cohen P 1987. Enzymes that process somatostatin precursors. J. Biol. chem. 262:9615-9620.

Gomez-Pan A, Reed JD, Albinus M, Shaw B, and Hall R 1975. Direct inhibition of gastric acid and pepsin secretion by growth-hormone release-inhibiting hormone in cats. Lancet 19;1(7912):888-890.

Gonzalez GA, and Montminy MR 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59:675-680.

Goodman RH, Aron DC, Roos BA 1983. Rat pre-prosomatostatin. Structure and processing by microsomal membranes. J Biol Chem 258(9):5570-5573.

Gossard F, Dihl F, Pelletier G, Dubois PM, and Morel G 1987. In situ hybridization to rat brain and pituitary gland of growth hormone cDNA. Neurosci Lett 79:251-256

Grau EG, Nishioka RS, Young G, and Bern HA 1985. somatostatin-like immunoreactivity in the pituitary and brain of three teleost fish species: somatostatin as a potential regulator of prolactin cell function. Gen Comp Endocrin 59:350-357.

Guo F, Beaudet A, and Tannenbaum GS 1996. The effect of hypophysectomy and growth hormone replacement on sst1 and sst2 somatostatin receptor subtype messenger ribonucleic acids in the arcuate nucleus. Endocrinology 137(9):3928-3935.

Hartman ML, Veldhuis JD, Johnson ML, Lee MM, Alberti KG, Samojlik E, and Thorner MO 1992. Augmented growth hormone (GH) secretory burst frequency and amplitude mediate enhanced GH secretion during a two-day fast in normal men. J Clin Endocrin Metab. 74(4):757-765.

Harvey S 1993. Growth hormone secretion in poililotherms and homeotherms. In "The Endocrinology of Groth, Development, and Metabolism in Vertebrates" (M.P. Schreibman, c.G. Scanes, P.K.T. Pang, Eds.) pp. 151-182. Acedemic Press, New York.

Harvey S, Scanes CG, and Daughaday WH 1995. "Growth Hormone" CRC Press USA.

Hasegawa O, Sugihara H, Minami S, and Wakabayashi I 1992. Masculinization of growth hormone (GH) secretory pattern by dihydrotestosterone is associated with augmentation of hypothalamic somatostatin and GH-releasing hormone mRNA levels in ovariectomized adult rats. Peptides 13(3):475-481.

Hellman B, and Lernmark A 1969. Evidence for an inhibitor of insulin release in the pancreatic islets. Diabetologia 5(1):22-24.

Herbison AE, Skinner DC, Robinson JE, and King IS 1996. Androgen receptor-immunoreactive cells in ram hypothalamus: distribution and co-localization patterns with gonadotropin-releasing hormone, somatostatin and tyrosine hydroxylase. Neuroendocrin 63(2):120-131.

Ho LT, Lam HC, Wang JT, Tang KT, Perng JC, Liu YF, and Chen RL 1986. Establishment of a radioimmunoassay of somatostatin in human plasma and in rat hypothalamus.Chin J Physiol 29:91-103.

Hobart P, Crawford R, Shen L, Pictet R, and Rutter WJ 1980. Cloning and sequence analysis of cDNAs encoding two distinct somatostatin precursors found in the endocrine pancreas of anglerfish. Nature 288(5787):137-141.

Holloway AC, and Leatherland JF 1997. Effect of gonadal steroid hormones on plasma growth hormone (GH) concentrations in sexually immature rainbow trout, Oncorhynchus mykiss. Gen. Comp. Endocrin. 105: 246-254.

Holloway AC, Sheridan MA, and Leatherland JF. 1998. Estradiol inhibits plasma somatostatin 14 (SS-14) levels and inhibits the response of somatostrophic cells to SS-14 challenge in vitro in rainbow trout, Oncorhynchus mykiss. Gen. Comp. Endocrin 106:407-414.

Jeandel L, Okuno A, Kobayashi T, Kikuyama S, Tostivint H, Lihrmann I, Chartrel N, Conlon JM, Fournier A, Tonon MC, and Vaudry H 1998. Effects of the two somatostatin variants somatostatin-14 and [Pro2, Met13]somatostatin-14 on receptor binding, adenylyl cyclase activity and growth hormone release from the frog pituitary. J Neuroendocrinol 10:187-192.

Johansson O, Hokfelt T, and Elde RP 1984. Immunohistochemical distribution of somatostatin-like immunoreactivity in the central nervous system of the adult rat. Neurosci. 13:265-339.

Jordan V, Dieguez C, Lafaffian I, Rodriguezarnao MD, Gomez-Pan A, Hall R, and Scanlon MF 1986. Influence of dopaminergic, adrenergic and cholinergic blockade and TRH administrationon GH responses to GRF 1-29. Clin Endocrin 24:291-298.

Kagawa H, Yourn G, and Nagahama Y, 1983. Changes in plasma steroid hormone levels during gonadal maturation in female goldfish, Carassius auratus. Bull. Japan. Soc. Sci, Fish. 49:1783-1787.

Kah O, Chambolle P, Dubourg P, and Dubois MP 1982. Localisation immunocytochimique de la somatostatine dans le cerveau anterieur et l'hypophsye de deux teleosteen, le cyprin (Carassius auratus) et Gambusia sp. CR Acad. Sci 294:519-524.

Kah O, Dubourg P, Onteniente B, Geffard M, and Calas A 1986. The dopaminergic innervation of the goldfish pituitary. An immunocytochemical study at the electron-microscope level using antibodies against dopamine. Cell Tissue Res 244(3):577-582.

Kah O, Dulka JG, Duboug P, Thibault J, and Peter RE 1987. Neuroanatomical substrate for the inhibition of gonadotropin secretion in goldfish: Existence of a dopaminergic preoptico-hypophyseal pathway. Neuroendocrin 45: 451-458.

Kakizawa S. Kaneko T, Ogasawara T, and Hirano T. 1995. Changes in plasma somatolactin levels during spawning migration of chum salmon (Oncorhynchus Keta). Fish physiol. Biochem 14: 93-101.

Katakami H, Berelowitz M, Marbach M, and Frohman LA 1985. Modulation of somatostatin binding to rat pituitary membranes by exogenously administered growth hormone. Endocrinology 117:557-560.

Kim MH, Oka Y, Amano M, Kobayashi M, Okuzawa K, Hasegawa Y, Kawashima S, Suzuki Y, and Aida K 1995. Immunocytochemical localization of sGnRH and cGnRH-II in the brain of goldfish, Carassius auratus. J Comp Neurol 356(1):72-82

Kimura N, Tomizawa S, Arai KN, and Kimura N 1998. Chronic treatment with estrogen up-regulates expression of sst2 messenger ribonucleic acid(mRNA) but down-regulates expression of sst5 mRNA in rat pituitaries. Endocrinology 139:1573-1580.

Kitajima N, Chihara K, Abe H, Okimura Y Fujii Y, Sato M, Shakutsui S, Watanabe M, and Fujita T 1989. Effects of dopamine on immunoreactive groth hormone-releasing factor and somatostatin secretion from rat hypothalamic slices perifused in vitro. Endocrinology 124:69-76.

Knuth UA, Sikand GS, Casanueva FF, and Friesen HG 1983. Changes in somatostatin-like activity in discrete areas of the rat hypothalamus during different stages of proestrus and diestrus and their relation to serum gonadotropin, prolactin, and growth hormone levels. Endocrinology 112:1506-1511.

Kobayashi M, Aida K, Hanyu I. 1986. Annual changes in plasma levels of gonadotropin and steroid hormones in goldfish. Bull Japan. Soc ci Fish 52:755.

Kraicer J, Cowan JS, Sheppard MS, Lussier B, and Moor BC 1986. Effect of somatostatin withdrawal and growth hormone (GH)-releasing factor on GH release in vitro: amount available for release after disinhibition. Endocrinology 119(5):2047-2051

Kwong P, and Chang JP 1997. Somatostatin inhibition of growth hormone release in goldfish: possible targets of intracellular mechanisms of action. Gen Comp Endocrin 108(3):446-456.

Lago F, Senaris RM, Emson PC, Dominguez F, and Diequez C 1996. Evidence for the involvement of non-androgenic testicular factors in the regulation of hypothalamic somatostatin and GHRH mRNA levels. Mol. Brain. Res. 35:220-226.

Lai ZN, Emtner M, Roos P, and Nyberg F 1991 Characterization of putative growth hormone receptors in human choroid plexus. Brain Res 546:222-226.

Lal S, Vasava Nair NP, Thavundayil JX, Tawar V, Quirion R, and Guyda H 1991. Stereospecificity of the dopamine receptor mediating the frowth hormone response to apomorphine in man. J Neural transm 85:157-164.

Leidy JW Jr, McDermott MT, and Robbins RJ 1990. Effect of hypophysectomy and growth hormone administration on hypothalamic growth hormone-releasing hormone and somatostatin content: relationship to age-related growth rate. Neuroendocrin 51:400-405.

Lepage-Lezin A., Joseph-Bravo P, Devillier G, Benedetti L, Launay JM , Gomez S, and Cohen P 1991. Prosomatostatin is processed in the Golgi Apparatus of rat neural cells. J. Biol. chem. 266: 1679-1688.

Lewis BM, Dieguez C, Lewis M, Hall R, and Scanlon MF 1986. Hypothalamic D2 receptors mediate the preferential release of somatostatin-28 in response to dopaminergic stimulation. Endocrincrinology 119:1712-1720

Lin XW, Lin HR, Peter RE 1993. Growth hormone and gonadotropin secretion in the common carp (Cyprinus carpio L.): invitro interactions of gonadotropin-releasing hormone, somatostatin, and the dopamine agonist apomorphine. Gen Comp Endocrinol 89(1):62-71.

Lin XW, and Peter RE 1996. Expression of salmon gonadotropin-releasing hormone (GnRH) and chicken GnRH-II precursor messenger ribonucleic acids in the brain and ovary of goldfish. Gen Comp Endocrinol 101(3):282-296.

Lin XW, and Peter RE 1997. Cloning and expression pattern of a second [His5Trp7Tyr8]gonadotropin-releasing hormone (chicken GnRH-H-II) mRNA in goldfish: evidence for two distinct genes. Gen Comp Endocrinol 107(2):262-272.

Lin XW, Otto CJ, and Peter RE. Evolution of Neuroendocrine Peptide Systems: Gonadotropin-Releasing Hormone and Somatostatin. Comp Biochem. Physiol. In press.

Linard B, Anglade I, Corio M, Navas JM, Pakdel G, Sligaut C, and Kah O 1996. Estrogen receptors are expressed in a subset of tyrosine hydroxylase-positive neurons of the anterior preoptic region in the rainbow trout. Neuroendocrin. 63:156-165.

Liao N, Vaudry H, and Pelletier G 1992. Neuroanatomical connections between corticotropin-releasing factor (CRF) and somatostatin (SRIF) nerve endings and thyrotropin-releasing hormone (TRH) neurons in the paraventricular nucleus of rat hypothalamus. Peptides 13(4):677-680.

Lucius R, and Mentlein R 1991. Degradation of the neuropeptide somatostatin by cultivated neuronal and glial cells. J Biol Chem 266(28):18907-18913.

Mahmoud SS, Moloney MM, and Habibi,HR. 1996. Cloning and sequencing of the goldfish growth hormone cDNA. Gen. Comp. Endocrin 101:139-144.

Maiter DM, Gabriel, SM Kornig JI, Russel WE, and Martin JB, 1990. Sexual differentiation of growth hormone feedback effects on hypothalamic growth hormone-releasing hormone and somatostaatin. Neuroendocrin 51:174-180.

Marchant TA, Dulka JG, and Peter RE 1988. Relationship between serum growth hormone levels and the brain and pituitary content of immunoreactive somatostatin in the goldfish, Carassius auratus. Gen. Comp. Endocrin 73: 458-468.

Marchant TA, and Peter RE 1986. Seasonal variations in body growth rates and circulating levels of growth hormone in the goldfish, Carassius auratus. J. Exp. Zool. 237: 231-239.

Marchant TA, and Peter RE 1989a. Hypothalamic peptides influencing growth hormone secretion in the goldfish, Carassius auratus. Fish phys. Biochem. 7:133-139.

Marchant TA, Dulka JG, and Peter RE 1989b.Relationship between serum growth hormone levels and the brain and pituitary content of immunoreactive somatostatin in the goldfish, Carassius auratus L. Gen Comp Endocrinol 73(3):458-468.

Marchant TA, Fraser RA, Andrews PC and Peter RE 1987. The influence of mammalian and teleost somatostatins on the secretion of growth hormone from goldfish (Carassius auratus L.) Pituitary fragments in vitro. Regul. Peptides, 17: 41-52.

Martinoli MG, Ouellet J, Rheaume E, and Pelletier G 1991. Growth hormone and somatostatin gene expression in adult and aging rats as measured byquantitative in situ hybridization. Neuroendocrin 54:607-615.

Matz SP, and Takahashi TT 1994. Immunohistochemical localization of thyrotropin-releasing hormone in the brain of chinook salmon (Oncorhynchus tshawytscha). J Comp Neurol 345(2):214-223.

McCarthy GF, Beaudet A, Tannenbaum GS 1992. Colocalization of somatostatin receptors and growth hormone-releasing factor immunoreactivity in neurons of the rat arcuate nucleus. Neuroendocrin 56(1):18-24.

Meister B, and Hulting AL 1987. Influence of coexisting hypothalamic messengers on growth hormone secretion from rat anterior pituitary cells in vitro. Neuroendocrin 46:387-394.

Melamed P, Eliahu N, Levavi-Sivan B, Ofir M, Farchi-Pisanty O, Rentier-Delrue F, Smal J, Yaron Z, and Naor Z 1995. Hypothalamic and thyroidal regulation of growth hormone in tilapia. Gen Comp Endocrin 97(1):13-30.

Melamed P, Gur G, Elizur A, Rosenfeld H, Sivan B, Rentier-Delrue F, and Yaron, Z. 1996. Differential effects of gonadotropin-releasing hormone, dopamine and somatostatin and their second messengers on the mRNA levels of gonadotropin IIb subunit and growth hormone in the teleost fish, tilapia. Neuroendocrin 64:320-328.

Mentlein R, and Dahms P 1994. Endopeptidases 24.16 and 24.15 are responsible for the degradation of somatostatin, neurotensin and other neuropeptides by cultivated rat cortical astrocytes. J. Neurochem. 62:27-36.

Merchenthaler I, Setalo, G, Csontos C, Petrusz P, Plerko B, and Negro-vilar A 1989. Combined retrograde tracing and immunocytochemical identification of luteinizing hormone-releasing hormone and somatostatin-containig neurons projuction to the median eminence of the rat. Endocrinology 125:2812-2821.

Meurnier H, and Labrie F, 1982. The dopamine receptor in the intermediate lobe of the rat pituitary gland is negatively coupled to adenylate cyclase. Life Sci 30: 963-968.

Minth CD, Taylor WL, Magazin M, Tavianini MA, Collier K, Weith HL, and Dixon JE 1982. The structure of cloned DNA complementary to catfish pancreatic somatostatin-14 messenger RNA. J Biol Chem 257(17):10372-10377.

Monsma FJ, Mahan LC, McVittie LD, Gerfen CR, and Sibley DR 1990. Molecular cloning and expression of a D1 dompamine receptor linked to adenylyl cyclase activation. Proc Natl Acad Sci USA 87: 6723-6727.

Montminy MR, Goodman RH, Horovitch SJ, and Habener JF 1984. Primary structure of the gene encoding rat preprosomatostatin. Proc Natl Acad Sci U S A 81(11):3337-3340.

Montminy MR, and Bilezikjian LM 1987. Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature 328(6126):175-178.

Montminy MR, Low MJ, Tapia-Arancibia L, Reichlin S, Mandel G, and Goodman RH 1986b. Cyclic AMP regulates somatostatin mRNA accumulation in primary diencephalic cultures and in transfected fibroblast cells. J Neurosci 6:1171-1176.

Montminy MR, Sevarino KA, Wagner JA, Mandel G, Goodman RH 1986a. Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc Natl Acad Sci U S A 83:6682-6686.

Moore C A, Kittilson JD, Dahl S K, and Sheridan M A. 1995. Isolation and characterization of a cDNA encoding for preprosomatostatin containing [Tyr7 Gly10]-somatostaton-14 from the endocrine pancreas of rainbow trout, Oncorhynchus mykiss. Gen. Comp Endocrin . 98: 253-261.

Morel A, Nicolas P, and Cohen P 1983. Evidence for a predominant form of Mr = 15,000 prosomatostatin in the mouse hypothalamus. Relationship with somatostatin-14 and -28. J Biol Chem 258(13):8273-8276.

Morrison MW, Davis SL, and Spicer LJ 1981. Age-associated changes in secretory patterns of growth hormone, prolactin and thyrotropin and the hormonal responses to thyrotropin-releasing hormone in rams. J Anim Sci 53:160-170.

Murao S, Sato M, Tamaki M, Niimi M, Ishida T, and Takahara J 1995. Gene expression of hypothalamic growth hormone (GH)-releasing hormone and somatostatin does not correlate with pulsatile secretion of GH in the adult mouse. Res. Comm. Mol. Path. Pharm. 89:269-276.

Nagahama Y 1994. Endocrine regulation of gametogenesis in fish. Intl. J. Dev. Biol. 38:217-229.

Namba H, Morita S, and Melmed, S 1989. Insulin-like grwoth factor-I action on growth hormone secretion and messenger ribonucleic acid levels: Interactions with somatostatin. Endocrinology : 24: 1794-1799.

Navas JM, Anglade I, Bailhache T, Pakdel F, Breton B, Jego P, and Kah O 1995. Do gonadotrophin-releasing hormone neurons express estrogen receptors in the rainbow trout? A double immunohistochemical study. J Comp Neurol 363:461-474

Nishi M, Moverus B, Bukovskaya O, Takahashi A, and Kawauchi H 1995. Isolation and characterization of [Pro2] somatostatin-14 and melanotropins from russian sturgeon, Acipenser gueldenstaedi brandt. Gen. Com. Endocrin. 99: 6-12.

Niznik HB 1987. Dopamine receptors: Molecular structure and function. Mol Cell Endocrinol. 54:1-22.

O'Carroll AM, and Krempels K 1995. Widespread distribution of somatostatin receptor messenger ribonucleic acids in rat pituitary. Endocrinology 136(11):5224-5227.

Ohno, S., Wold, U and Atkin NB 1968. Evolution from fish to mammals by gene duplication. Hereditas 59:169-187

Olivereau M, Ollevier F, Vandesande F, and Olivereau J. 1984a. Somatostatin in the brain and the pituitary of some teleosts Immunocytochemical identification and the effect of starvation. Cell Tiss. Res. 238: 289-296.

Olivereau M, Ollevier, F, Vandesand F, and Veronck M, 1984b. Immunocytochemical identification of CRF-like and SRIF-like peptides in the brain and pituitary of cyprinid fish. Cell tissue Res 237:379-382.

Olivereau M, and Olivereau JM 1991. Immunocytochemical localization of a galanin-like peptidergic system in the brain and pituitary of some teleost fish. Histochemistry 96(4):343-354.

Pacold ST, Kirsteins L, Hojvat S, and Lawrence AM 1978. Biologically active pituitary hormones in the rat brain amygdaloid nucleus. Science 199:804-806.

Pakdel P, LeGac F, Le Goff P, and Valotaire Y 1990. Full length sequence and in vitro expression of rainbow trout estrogen recetpor cDNA. Mol Cell Endocrinol. 71: 195-204.

Pankhurst RJ 1986. A package of computer programs for handling taxonomic databases. Comput Appl Biosci 2(1):33-39

Patel YC, and Wheatley T 1983. In vivo and in vitro plasma disappearance and metabolism of somatostatin-28 and somatostatin-14 in the rat. Endocrinology 112:220-225.

Patel YC, Wheatley T, and Ning C. 1981. Multiple forms of immunoreactive somatostatin: comparison of distribution in neural and nonnueral tissues and portal plasma of the rat. Endocrinology 109:1943-1949.

Pekary AE, Yamada T, Sharp B, Bhasin S, Swerdloff RS, and Hershman JM 1984. Somatostatin-14 and -28 in the male rat reproductive system. Life Sci 34:939-945.

Pellegrini E, Bluet-Pajot MT, Mounier F, Bennett P, Kordon C, and Epelbaum, J 1996 Central administration of a growth hormone (GH) receptor mRNA antisense increases GH pulsatility and decreases hyupothalamic somatostatin expression in rats. J. Neurosci. 16:8140-8148.

Peng C, and Peter RE 1997. Neuroendocreine regulation of growth hormone secretion and growth in fish. Zool. Stud. 36:79-89.

Pesek MJ, and Sheridan MA 1996. Fasting alters somatostatin binding to liver membranes of rainbow trout (Oncorhynchus mykiss). J Endocrinol 150(2):179-186.

Peter RE, Yu KL, Marchant TA and Rosenblum PM 1990. Direct neural regulation of the teleost adenhypophysis. J Exp. Zool (Suppl.) 4: 84-89.

Peter RE, and Marchant, TA 1995. The endocrinology of growth in carp and related species. Aquaculture 129:299-321.

Peyon P, Lin XW, Himick BA, Peter RE 1998. Molecular cloning and expression of cDNA encoding brain preprocholecystokinin in goldfish. Peptides 19(2):199-210

Pickavance, LC, Staines WA, and Fryer JN 1992. Distribution and colocalization of neuropeptide Y and somatostatin in the goldfish brain. J Chem Neuroant 5:221-233.

Plisetskaya EM, Pollock HG, Rouse JB, Hamilton JW, Kimmel JR, Andrews PC, Gorbman A 1986. Characterization of coho salmon (Oncorhynchus kisutch) islet somatostatins. Gen Comp Endocrin 63(2):252-263.

Plotsky PM 1991. Hypothalamic secretion of somatostatin and growth hormone-releasing factor into thehypophysial-portal circulation is reduced in streptozotocin diabetic male rats. Neuroendocrin 53:433-438.

Rabbani SN, and Patel YC 1990. Peptides derived by processing of rat prosomatostatin near the amino-terminus characterization, tissue distribution, and release. Endocrinology 126:2054-2061.

Rao PD, Murthy CK, Cook H, and Peter RE 1996. Sexual dimorphism of galanin-like immunoreactivity in the brain and pituitary of goldfish, Carassius auratus. J Chem Neuroanat 10(2):119-135.

Rao SD, Rao PD, and Peter RE 1996. Growth hormone-releasing hormone immunoreactivity in the brain, pituitary, and pineal of the goldfish, Carassius auratus. Gen Comp Endocrin 102(2):210-220.

Ravazzola M, Benoit R, Ling N, and Orci L 1989. Prosomatostatin-derived antrin is present in gastric D cells and in portal blood. J Clin Invest 83:362-366.

Reichlin S 1983. Somatostatin. N Engl J Med 309(24):1495-1501.

Reisine T 1995. Somatostatin. Cell Mol Neurobiol 15(6):597-614.

Reisine I and Bell GI 1995. Molecular biology of somatostatin receptors. Endocrine Rev. 16:427-442.

Richardson SB, Nguyen T, and Hollander CS 1983. Dopamine stimulates somatostatin release from perifused hypothalamic cells. Am J Physiol 244:E560-E566.

Salbert G, Atteke C, Bonnec G, and Jego P 1993. Differential regulation of the estrogenn recetpor mRNA by estrdiol in the trout hypothalamus and pituitary. Mol Cell Endocrinol 96:177-182.

Salbert G, Bonnec G, Le Goff P, Boujard D, Valotair Y, and Jego P 1991. Localization of the estradiol mRNA in the forebrain of the rainbow trout. Mol Cell endocrinol 78: 173-180.

Sato M, and Frohman LA 1993. Differential effects of central and peripheral administration of growth hormone (GH) and insulin-like growth factor on hypothalamic GH-releasing hormone and somatostatin gene expression in GH-deficient dwarf rats. Endocrinology 133:793-799.

Sato M, Chihara K, Kita T, Kashio Y, Okimura Y, Kitajima N, and Fujita T 1989. Physiological role of somatostatin-mediated autofeedback regulation for growth hormone:importance of growth hormone in triggering somatostatin release during a trough period ofpulsatile growth hormone release in conscious male rats. Neuroendocrin 50:139-151.

Saunders A, Terry LC, Audet J, Brazeau P and Martin JB 1976. Dynamic studies of growth hormone and prolactin secretion in the female rat. Neuroendocrin 21:193-203.

Schally AV, Coy DH, and Meyers CA 1978. Hypothalamic regulatory hormones. Annu Rev Biochem 47:89-128.

Shen LP, Pictet RL, Rutter WJ 1982. Human somatostatin I: sequence of the cDNA. Proc Natl Acad Sci U S A 79(15):4575-4579.

Shen LP, and Rutter WJ 1984. Sequence of the human somatostatin I gene. Science 224(4645):168-171.

Sheppard M, Shapiro B, Pimstone B, Kronheim S, Berelowitz M, and Gregory M 1979. Metabolic clearance and plasma half-disappearance time of exogenous somatostatin in man. J Clin Endocrinol Metab 48:50-53.

Shoelson SE, Polonsky KS, Nakabayashi T, Jaspan JB, and Tager HS 1986. Circulating forms of somatostatinlike immunoreactivity in human plasma. Am J Physiol 250:E428-E434.

Sidhu A, Kimura K, Uh M, White BH, and Patel S 1998. Multiple coupling of human D5 dopamine receptors to guanine nucleotide binding proteins Gs and Gz. J Neurochem 70:2459-2467.

Sinisi AA, Bellastella A, Prezioso D, Nicchio MR, Lotti T, Salvatore M, and Pasquali D 1997. Different expression patterns of somatostatin receptor subtypes in cultured epithelial cells from human normal prostate and prostate cancer. J Clin Endocrinol Metab 82:2566-2569.

Slama A, Videau C, Kordon C, and Epelbaum J 1992. Estradiol regulation of somatostatin receptors in the arcuate nucleus of the female rat. Neuroendocrin 56(2):240-245.

Slama A, Bluet-Pajot MT, Mounier F, Videau C, Kordon C, and Epelbaum J 1993. 125I-somatostatin-labeled cells in the anterior arcuate nucleus mediate somatostatin effects on growth hormone but not prolactin secretion. Neuroendocrin58(2):178-184.

Sloley BD, Trudeau VL, Dulka JG, and Peter RE 1991. Selective depletion of dopamine in the goldfish pituitary caused by domperidone. Can J Physiol Pharmacol 69(6):776-781.

Somoza GM, and Peter RE 1991. Effects of serotonin on gonadotropin and growth hormone release from in vitro perifused goldfish pituitary fragments. Gen Comp Endocrin 82(1):103-110.

Sonntag WE, Forman LJ, Miki, N, Trapp JM, Gottschall PE, and Meites J 1982. L-Dopa restores amplitude of growth hormone pulses in old male rats to that observed in young male rats. Neuroendocrin 34:163-168.

Stacey NE, Mackenzie DS, Marchant TA, Kyle AL, and Peter RE 1984. Endocrine changes during natural spawning in the white sucker, Catostomus commersoni. I Conadotropin, growth hormone and throid hormones. Gen Comp Endocrin 56: 333-348.

Steiner RA, Illner P, Rolf AD, Toivola PTK, and Gale CC 1978. Noradrenergic and dopaminergic regulation of GH and prolactin in baboons. Neuroendocrin 26:15-31.

Stern WC, Miller M, Resnick O, and Morgane PJ 1975. Distribution of 125I-labeled rat growth hormone in regional brain areas and peripheral tissue of the rat. Am J Anat 144:503-507.

Stewart F, Goode JA, and Allen WR 1993. Growth hormone secretion in the horse: unusual pattern at birth and pulsatile secretion through to maturity. J Endocrinol 138:81-89.

Stewart JK, Goodner CJ, Koerker DJ, Gorbman A, Ensinck J, Kaufman M 1978. Evidence for a biological role of somatostatin in the Pacific hagfish, Eptatretus stouti. Gen Comp Endocrin 36(3):408-414.

Su CJ, White JW, Li WH, Luo CC, Frazier ML, Saunders GF and Chan L 1988. Structure and evolution of somatostatin genes. Molec. Endocrin. 2: 209-216.

Sumpeter JP, Lincoln RF, Bye, VJ, Carragher, JF, and Le Bail, PY 1991. Plasma growth hormone levels during sexual maturation in diploid and triploid rainbow trout (Oncorhynchus mykiss). Gen Comp Endocrin. 83: 103-110.

Tannenbaum GS, Guyda HJ, and Posner BI 1983. Insulin-like growth factors: a role in growth hormone negative feedback and body weight regulation via brain. Science 220:77-79.

Tannenbaum GS, Zhang WH, Lapointe M, Zeitler P, Beaudet A 1998. Growth hormone-releasing hormone neurons in the arcuate nucleus express both Sst1 and Sst2 somatostatin receptor genes. Endocrinology 139:1450-1453.

Tannenbaum, GS, and Martin, JB 1976. Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. Endocrinology 98:562-570.

Tavianini MA, Hayes TE, Magazin MD, Minth CD, and Dixon JE 1984. Isolation, characterization, and DNA sequence of the rat somatostatin gene. J Biol Chem. 259(19):11798-11803.

Terry LC, Rorstad IP, and Martin JB 1980. The release of biologically and immunologically reactive somatostatin from perifused hypothalamic fragments. Endocrinology 107:794-800.

Tominaga T, Fukata J, Hayashi Y, Satoh Y, Fuse N, Segawa H, Ebisui O, Nakai Y, Osamura Y, and Imura H. 1992 Distribution and characterization of immunoreactive corticostatin in the hypothalamic-pituitary-adrenal axis. Endocrinology 130(3):1593-1598.

Torres I, Guaza C, Fernandez-Durango R, Borrell J, and Charro AL 1982. Evidence for a modulatory role of catecholamines on hypothalamic somatostatin in the rat. Neuroendocrin 35:159-162.

Trudeau VL, Sloley BD, and Peter RE 1993. Norepinephrine turnover in the goldfish brain is modulated by sex steroids and GABA. Brain Res 624(1-2):29-34.

Trudeau, VL, Somoza, GM, Nahorniak, CS, and Peter, RE. 1992. Interactions of estradiol with gonadotropin-releasing hormone and thryotropin-releasing hormone in the control of growth hormone secretion in the goldfish. Neuroendocrin. 56:483-490.

Trudeau VL, Sloley BD, Kah O, Mons N, Dulka JG, and Peter RE 1996. Regulation of growth hormone secretion by amino acid neurotransmitters in the goldfish (I): Inhibition by N-methyl-D, L-aspartic acid. Gen Comp Endocrinol 103(2):129-137.

Turman MA, O'Dorisio MS, O'Dorisio TM, Apple CA, and Albers AR 1997. Somatostatin expression in human renal cortex and mesangial cells. Regul Pept 68:15-21.

Uesaka T, Yano K, Yamasaki M, Nagashima K, and Ando M 1994. Somatostatin-related peptides isolated from the eel gut: effects on ion and water absorption across the intestine of the seawater eel. J Exp Biol 188:205-216.

Uesaka T, Yano K, Yamasaki M, and Ando M 1995. Somatostatin-, vasoactive intestinal peptide-, and granulin-like peptides isolated from intestinal extracts of goldfish, Carassius auratus. Gen. Comp. Endocrin 99: 298-306.

Vale W, Rivier C, Brazeau P, and Guillemin R 1974. Effects of somatostatin on the secretion of thyrotropin and prolactin. Endocrinology 95(4):968-977.

Vasavan Nair NPV, Lal S, Eugenio H,Lizondo E, Thavundayil JX, Wood PL, Etienne P, and Guyda H 1986. CCK-8 antagonizes apomorphrine-induced growth hormone secretion in normal subjects. Horm Metabol Res 18:53-55.

Vaudry H, Chartrel N and Conlon M 1992. Isolation of [Pro2, Met13]Somatostatin-14 and somatostatin 14 from the frog brain reveals the existence of a somatostatin gene family in a tetrapod. Biochem. Biophy. Res. Communication. 188:477-482

Veldhuis JD, Iranmanesh A, Ho KK, Waters MJ, Johnson ML, and Lizarralde G 1991. Dual defects in pulsatile growth hormone secretion and clearance subserve the hyposomatotropism of obesity in man. J Clin Endocrinol Metab 72:51-59.

Vigh-Teichmann I, Vigh B, Korf HW, and Oksche A 1983. CSF-contacting and other somatostatin immunoreactive neurons in the brains of *Anguilla anguilla*, *Phoxinus phoxinus* and *Salmo gairdneri* (Teleostei). Cell Tissue Res. 233:319-334.

Voight KH, Fehm HL, Lang RE, Walter R 1977. The effect of somatostatin and of prolyl-leucyl-glycinamide (MIF) on ACTH release in dispersed pituitary cells. Life Sci 21(5):739-745.

Wakabayashi I, Miyazawa Y, Kanda M, Miki N, Demura R, Demura H, and Shizume K 1977. Stimulation of immunoreactive somatostatin release from hypothalamic synatosomes by high (K+) and dopamine. Endocrin Japan 24:601-604.

Walter P, Gilmore R, and Blobel G 1984. Protein translocation across the endoplasmic reticulum. Cell 38(1):5-8.

Wang Y, Youson JH and Conlon J M 1993. Prosomatostatin-I is processed to somatostatin-26 and somatostatin-14 in the pancreas of the bowfin. Amia calva. Reg. Peptides 47: 33-39.

Wass JA 1988. Measurement of somatostatin. Horm Res 29(2-3):86-88.

West CR, Lookingland, KJ, and Tucker, HA 1997. Tegulation of growth hormone-releasing hormone and somatostatin from perifused, bovine hypothalamic slices. III. Reciprocal feedback between growth hormone-releasing hormone and somatostatin. Domest Anim Endocrinol 14:358-366.

Werner H, Koch Y, Baldino F Jr, and Gozes I 1988. Steroid regulation of somatostatin mRNA in the rat hypothalamus. J Biol Chem 263(16):7666-7671.

Widstrom AM, Matthiesen AS, Winberg J, and Uvnas-Moberg K 1989. Maternal somatostatin levels and their correlation with infant birth weight. Hum Dev 20:165-174.

Willoughby J O, Brogan M., and Kapor R 1989. Hypothalamic interconnections of somatostatin and growth hormone releasing factor neurons. Neuroendocrin 50:584-591.

Willoughby JO, and Day TA 1981. Central catecholamine depletion: effects on physiological growth hormone and prolactin secretion. Neuroendocrin 32:65-69.

Wong AOL, Chang JP, and Peter RE 1993a. Dopamine functions as a growth hormone-releasing factor in the goldfish, Carassius auratus. Am. J. physiol 264(Endocrinol. Metab. 27):E925-E932.

Wong AOL, Chang JP, and Peter RE 1992. Dopamine stimulates growth hormone release from the pituitary of goldfish, Carassius auratus, through the dopamine D1 receptors. Endocrinology 130:1201-1210.

Wong AOL, Change JP, and Peter RE 1993b. Characterization of D1 receptors mediating dopamine-stimulated growth hormone release from Pituitary cells of the goldfish, Carassius auratus. Endocrinology 133: 577-584.

Wong AOL, Leung MY, Shea WLC, Tse LY, Chang JP and Chow BKC 1998. Hypophyusiotropic action of pituitary adenylate cyclase activating Polypeptide (PACAP) in the goldfish: Immunohistochemical demonstration of PACAP in the pituitary, PACAP stimulation of growth hormone release form pituitary cells, and molecular cloning of pituitary type I PACAP receptor. Endocrinology In press.

Wood TL, Berelowitz M, Gelato MC, Roberts CT Jr, LeRoith D, Millard WJ, and McKelvy JF 1991. Hormonal regulation of rat hypothalamic neuropeptide mRNAs: effect of hypophysectomy and hormone replacement on growth-hormone-releasing factor, somatostatin and the insulin-like growth factors. Neuroendocrin 53:298-305.

Wright NM, Northington FJ, Miller JD, Veldhuis JD, and Rogol AD 1992. Elevated growth hormone secretory rate in premature infants: deconvolution analysis of pulsatile growth hormone secretion in the neonate. Pediatr Res 32(3):286-290.

Wu D, Chen C, Zhang J, Bower CY, and Clarke IJ. 1996. The effects of GH-releasing peptide-6 (GHRP-6) and GHRP-2 on intracellular adenosine 3', 5'-monophosphate (cAMP) levels and GH secretion in ovine and rat somatotrophs. J Endocrin 148:197-205.

Yada T, and Hirano T 1992. Inhibition of growth hormone synthesis by somatostatin in cultured pituitary of rainbow trout. J. Comp Physiol 162:575-580.

Zeitler P, Tannenbaum GS, Clifton DK, and Steiner RA 1991. Ultradian oscillations in somatostatin and growth hormone-releasing hormone mRNAs in the brains of adult male rats. Proc. Natl. Acad. Sci. USA. 88:8920-8924.

Zhu LJ, Krempels K, Bardin CW, O'Carroll AM, and Mezey E 1998. The localization of messenger ribonucleic acids for somatostatin receptors 1, 2, and 3 in rat testis. Endocrinology 139:350-357.

Zou JJ, Trudeau VL, Cui Z, Brechin J, Mackenzie K, Zhu Z, Houlihan, DF, and Peter RE 1997. Estradiol stimulates growth hormone production in female goldfish. Gen Comp Endocrinology 106:102-112

APPENDIX 1a

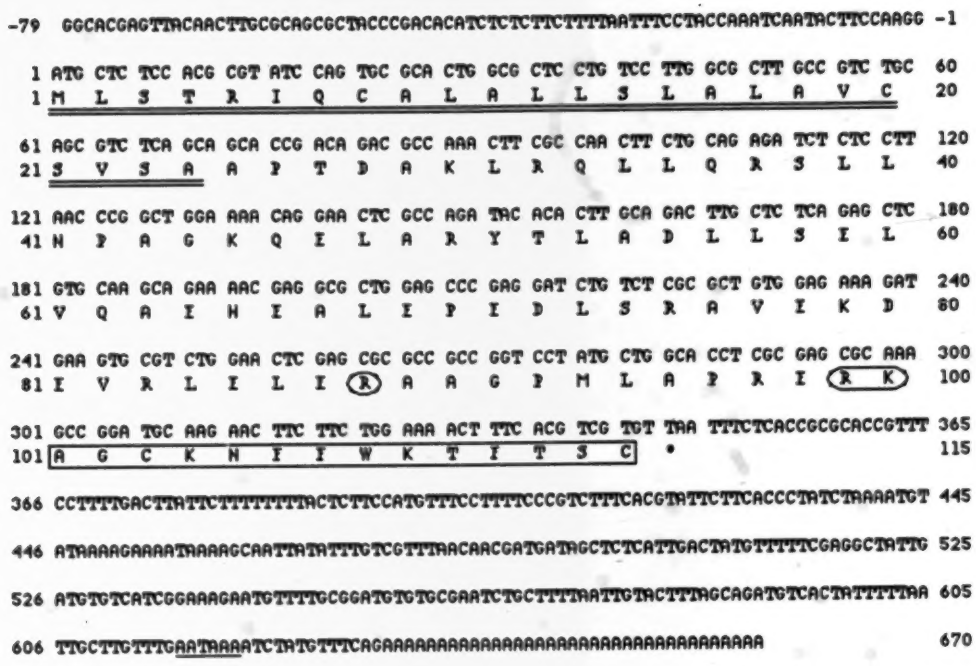
-79 GGCACGAGTTACAACTTGCGCAGCGCTACCCGACACATCTCTCTTCTTTTAATTCCTACCAAATCAATACTTCCAAGG -1
1 ATG CTC TCC ACG CGT ATC CAG TGC GCA CTG GCG CTC CTG TCC TTG GCG CTT GCC GTC TGC 60
1 M L S T R I Q C A L A L L S L A L A V C 20
61 AGC GTC TCA GCA GCA CCG ACA GAC GCC AAA CTT CGC CAA CTT CTG CAG AGA TCT CTC CTT 120
21 S V S A A P T D A K L R Q L L Q R S L L 40
121 AAC CCG GCT GGA AAA CAG GAA CTC GCC AGA TAC ACA CTT GCA GAC TTG CTC TCA GAG CTC 180
41 N P A G K Q E L A R Y T L A D L L S E L 60
181 GTG CAA GCA GAA AAC GAG GCG CTG GAG CCC GAG GAT CTG TCT CGC GCT GTG GAG AAA GAT 240
61 V Q A E N E A L E P E D L S R A V E K D 80
241 GAA GTG CGT CTG GAA CTC GAG CGC GCC GCC GGT CCT ATG CTG GCA CCT CGC GAG CGC AAA 300
81 E V R L E L E R A A G P M L A P R E R K 100
301 GCC GGA TGC AAG AAC TTC TTC TGG AAA ACT TTC ACG TCG TGT TAA TTTCTCACCGCGCACCGTTT 365
101 A G C K N F F W K T F T S C • 115
366 CCTTTTGACTTATTCTTTTTTTTACTCTTCCATGTTTCCTTTTCCCGTCTTTCACGTATTCTTCACCCTATCTAAAATGT 445
446 ATAAAAGAAAATAAAAGCAATTATATTTGTCGTTTAACAACGATGATAGCTCTCATTGACTATGTTTTTCGAGGCTATTG 525
526 ATGTGTCATCGGAAAGAATGTTTTGCGGATGTGTGCGAATCTGCTTTTAATTGTACTTTAGCAGATGTCACTATTTTTAA 605
606 TTGCTTGTTTGAATAAAATCTATGTTTCAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 670

Figure 1a: The cDNA sequence for PSS-I1 isolated from the brain of goldfish by 3' and 5'-RACE and library screening (Wei X-L and Peter, unpublished). Double underline is the proposed signal peptide sequence, circled are mono and dibasic cleavage sites for possible mature peptide synthesis, Boxed is the proposed mature peptide produced (SS-14), and single underline is the polyadenilation signal sequence.

APPENDIX 1b

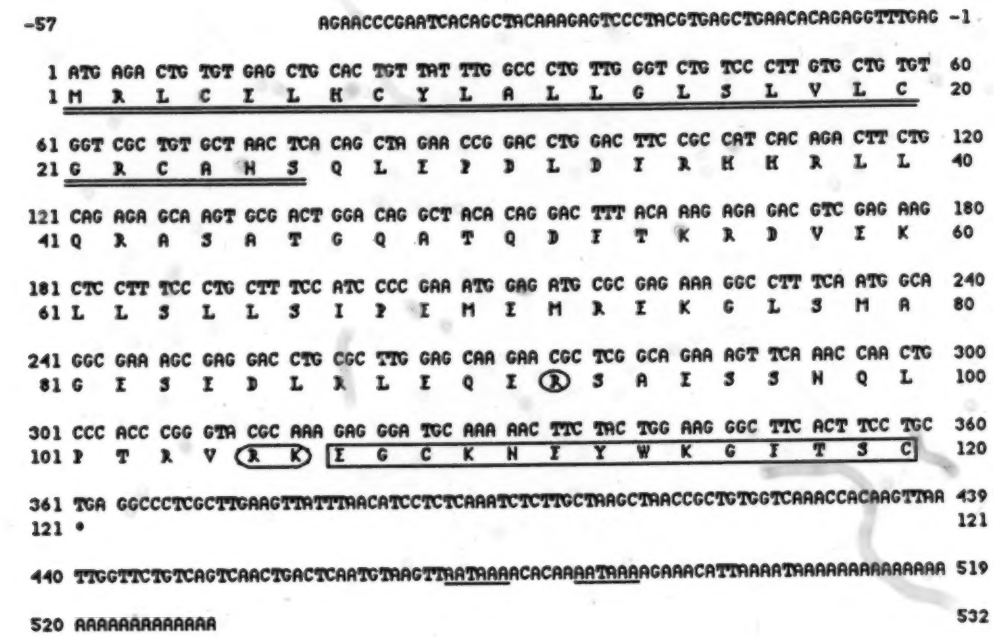
-57 AGAACCCGAATCACAGCTACAAAGAGTCCCTACGTGAGCTGAACACAGAGGTTTGAG -1
1 ATG AGA CTG TGT GAG CTG CAC TGT TAT TTG GCC CTG TTG GGT CTG TCC CTT GTG CTG TGT 60
1 M R L C E L H C Y L A L L G L S L V L C 20
61 GGT CGC TGT GCT AAC TCA CAG CTA GAA CCG GAC CTG GAC TTC CGC CAT CAC AGA CTT CTG 120
21 G R C A N S Q L E P D L D F R H H R L L 40
121 CAG AGA GCA AGT GCG ACT GGA CAG GCT ACA CAG GAC TTT ACA AAG AGA GAC GTC GAG AAG 180
41 Q R A S A T G Q A T Q D F T K R D V E K 60
181 CTC CTT TCC CTG CTT TCC ATC CCC GAA ATG GAG ATG CGC GAG AAA GGC CTT TCA ATG GCA 240
61 L L S L L S I P E M E M R E K G L S M A 80
241 GGC GAA AGC GAG GAC CTG CGC TTG GAG CAA GAA CGC TCG GCA GAA AGT TCA AAC CAA CTG 300
81 G E S E D L R L E Q E R S A E S S N Q L 100
301 CCC ACC CGG GTA CGC AAA GAG GGA TGC AAA AAC TTC TAC TGG AAG GGC TTC ACT TCC TGC 360
101 P T R V R K E G C K N F Y W K G F T S C 120
361 TGA GGCCCTCGCTTGAAGTTATTTAACATCCTCTCAAATCTCTTGCTAAGCTAACCGCTGTGGTCAAACCACAAGTTAA 439
121 * 121
440 TTGGTTCTGTCAGTCAACTGACTCAATGTAAGTTAATAAAACACAAAATAAAAGAAACATTAAAATAAAAAAAAAAAAA 519
520 AAAAAAAAAAAA 532

Figure 1b: The cDNA sequence for PSS-II isolated from the brain of goldfish by 3' and 5'-RACE and library screening (Wei X-L and Peter, unpublished). Double underline is the proposed signal peptide sequence, circled are mono and dibasic cleavage sites for possible mature peptide synthesis, boxed is the proposed mature peptide produced (SS-14), and single underline are the two polyadenilation signal sequences.

APPENDIX 2

SPECIES	PRECURSOR I					
Mammalian-28 & 14	SANSN	PAMAP	RERKA	GCKNF	FWKTF	TSC
Anglerfish-14	AASGG	PLLAP	RERKA	GCKNF	FWKTF	TSC
Bowfin-26	SAN**	PALAP	RERKA	GCKNF	FWKTF	TSC
Catfish-14			A	GCKNF	FWKTF	TSC
Lamprey						
Geotria-33	AVQEAGG A**AM	PPPGQ	RDRKA	GCKNF	FWKTF	SSC
Lampetra-35	AAAAPGA AGGAQ	LPLGN	RERKA	GCKNF	FWKTF	SSC
Petromyzon-14			A	GCKNF	FWKTF	TSC
Hagfish-34	AAERPRQ DG*QV	HEPPG	RERKA	GCKNF	FWKTF	TSC
	PRECURSOR II					
Anglerfish-28	SVDST	NNLPP	RERKA	GCKNF	YWKGF	TSC
Eel-25	SVD**	*NQQG	RGRKA	GCKNF	YWKGF	TSC
Flounder-28	IEPP	NNLPP	RERKA	GCKNF	YWKGF	TSC
Salmon-25	SVD**	*NLPP	RERKA	GCKNF	YWKGF	TSC
Catfish-22		NDNTV	NSKPL	NCMNY	FWKSR	TAC
Petromyzon-34 AAAVAGS	PQ*QL	LPLGQ	RERKA	GCKNF	YWKGF	TSC
Rainbow trout-28	SVGNP	NNLPP	RERKA	GCKNF	YWKGF	TSC
Sculpin-28	SVDST	NNIPL	RERKA	GCKNF	YWKGF	TSC
Goldfish Intestine-28	SVESS	DHLPA	RERKA	GCKNF	YWKGF	TSC

TABLE A2-1: Comparison of SS amino acid sequences between species. "*"Sequences are arrange maximum identity, it is assumed therefore that some amino acids are deleted (marked with*).